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TOXICOLOGIC AND ANALYTICAL STUDIES WITH T-2
AND RELATED TRICHOTHECENE MYCOTOXINS

SECOND ANNUAL PROGRESS REPORT
FOR THE PERIOD MARCH 11, 1983 THROUGH AUGUST 15, 1984

AUTHORS

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SUBMITTED SEPTEMBER 4, 1984

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ABSTRACT                 Second Annual Report DAMD 17-82-C-2179

Swine have been used as a model for assessing the effects of exposure to T-2 toxin. Acute T-2 toxicosis is a cardiovascular shock syndrome characterized by reductions in cardiac output and blood pressure, and increased plasma concentrations of epinephrine, norepinephrine, thromboxane $B_2$, 6-keto-PGF $_{1 alpha}$, and lactate. An initial leukocytosis is followed by a leukopenia. Serum bound calcium concentrations decrease while magnesium, phosphorus and potassium increase. Blood flow to the heart, brain, kidneys, spleen and stomach decreases, while blood flow to the adrenal glands, liver and intestines increases.

Topical application of T-2 toxin in swine produces a severe necrotizing dermatitis with the healing process beginning on day 7. Morphologic changes in internal organs are minimal. Responses of purified peripheral blood lymphocytes to the T-cell mitogens, phytohemagglutinin and concanavalin A, are decreased between days 20 and 31 after dosing. Neutrophilia and fever are apparent during the first 2 weeks of exposure. Body weights of treated pigs decrease after the first week. There are significant reductions in serum albumin, alkaline phosphatase and glucose but increases in serum globulin in the first 2 weeks.

Sublethal intravenous injections of T-2 toxin produce heart and pancreatic lesions in addition to the well-documented radiomimetic lesions. Grossly, there are subendocardial hemorrhages, pin-point white foci in the myocardium and pancreatic edema. Microscopic and ultrastructural changes in the heart include myofiber degeneration, vacuolization, necrosis and mineralization with formation of hypercontraction bands. Pancreatic changes consist of acinar degeneration and necrosis which progress to a diffuse suppurative necrotizing pancreatitis.

Techniques have been developed for using a closed-system inhalation procedure for monitoring dose-response effects of T-2 aerosol exposures.

Numerous potential therapeutic agents have been tested for efficacy against T-2 toxicosis. Only methylprednisolone and a combination of phenoxybenzamine plus Ringer's solution have apparent benefit. A new "superactive" activated charcoal, however, appears to have excellent adsorptive capacity for T-2 toxin and in preliminary studies was very effective in preventing T-2 toxicosis in rats. Since a major portion of absorbed T-2 toxin and its metabolites are excreted into the gastrointestinal tract, activated charcoal may be beneficial for treatment, as well as prevention, of trichothecene toxicoses, regardless of the route of exposure.

T-2 toxin once absorbed is primarily metabolized by the liver. The major metabolites include 3'OH T-2, HT-2, and 3'OH HT-2 and their glucuronide conjugates, all of which are primarily excreted into the urine and gastrointestinal tract.

TOXICOLOGIC AND ANALYTICAL STUDIES WITH T-2
AND RELATED TRICHOTHECENE MYCOTOXINS


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SUBMITTED SEPTEMBER 4, 1984

SUMMARY OF PROGRESS

I.  Swine Pathophysiologic Studies

A.  Hemodynamics, Vasoactive Mediators, Hematology and Serum Biochemistries

T-2 toxin was given as a single intravascular dose at either 0.6 or 4.8 mg/kg to different groups of 50 kg, female swine. Shock was characterized by reductions in cardiac output and blood pressure, and increased plasma concentrations of epinephrine, norepinephrine, thromboxane $B_2$, 6-keto-PGF$_{1 \, alpha}$ and lactate. Total peripheral resistance was unchanged in the high-dose group but decreased in the low-dose group. Pulmonary vascular resistance increased in both groups. Decreases occurred in arterial pH and arterial oxygen partial pressure. No alterations occurred in plasma concentrations of histamine or serotonin.

Blood samples were taken at hourly intervals for determination of concentrations or activities of the following substances in serum or plasma: creatinine, blood urea nitrogen, inorganic phosphorus, total calcium, ultrafilterable calcium, magnesium, sodium, potassium, chloride, total protein, albumin, cholesterol, glucose, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin. Coagulation analyses included prothrombin time, partial thromboplastin time, activated coagulation time, and fibrin degradation products. Red blood cell, white blood cell, and platelet counts, hemoglobin concentrations and hematocrits, were determined from whole blood samples.

An initial leukocytosis was followed by a leukopenia. The numbers of red blood cells, hemoglobin concentration, and hematocrit were increased. Nucleated red blood cells were seen in the blood smears. The serum concentration of bound calcium decreased, while phosphorus, magnesium and potassium increased. Clinical screening tests detected no evidence of a coagulopathy.

B. **Systemic Distribution of Blood Flow During T-2 Toxicosis**

Additional studies were conducted with swine dosed intravascularly with T-2 toxin at 0, 0.6 and 2.4 mg/kg, to determine if alterations occurred in blood flow to vital tissues of the body. Hemodynamic measurements were also performed.

Cardiac output declined in both treated groups of animals for up to 3 hours, after which the high-dose group continued to decline and the low-dose group began to rise. Aortic mean blood pressure of the high-dose group did not begin decreasing until after 1 1/2 hours.

Myocardial and brain blood flow had similar patterns of changes following T-2 administration. Both decreased following administration of 2.4 mg/kg and both had marked fluctuations in blood flow following administration of 0.6 mg/kg. Blood flow to the adrenals, liver, small intestine and large intestine of animals dosed with T-2 toxin was increased when compared to pre-dosing and vehicle control values. Renal, splenic and stomach blood flows decreased following T-2 administration, with blood flow to the spleen being the most severely compromised.

The study is still in progress and once it is completed and statistical analyses are performed much more meaningful results are expected.

C. Development of a Closed-System Inhalation Procedure for Studying Dose-
   Response Effects of T-2 Aerosol Exposures

Considerable effort has been invested in developing a system for studying the effects in swine, of inhaling an aerosol of T-2 toxin. Techniques have been worked out and protocols developed for the following: 1) animal restraint and monitoring, 2) toxin handling and administration, 3) aerosol generation and clean-up, and 4) aerosol characterization. These efforts have resulted in the ability to produce clinically apparent signs of T-2 toxicosis by the inhalation route of exposure. There are continuing efforts to increase

1

the efficiency of toxin delivery. Although we are able to estimate the dose of toxin retained by the pigs, a more reproducible and accurate method, using a radioactive isotope, is under development. This technique will allow more meaningful assessment of dose-response relationships and comparisons with the other routes of administration.

D. Testing of Potential Therapeutic Agents for T-2 Toxicosis

Rats and swine have been used for initial screening of a number of agents for their efficacy in treating acute T-2 toxicosis. In rats, methylprednisolone (a glucocorticosteroid), ascorbic acid, adenosine triphosphate alone and in combination with magnesium chloride, dazemgrel (a thromboxane synthetase inhibitor), trichodermin (a trichothecene mycotoxin), naloxone (an opioid antagonist), diltiazem (a slow calcium channel blocker), aprotinin (a protease inhibitor), N-acetylcysteine (a glutathione precursor) and dimethyl sulfoxide have been tested. In swine, the general alpha-adrenergic blocker, phenoxybenzamine, was combined with Ringer's solution and evaluated for potential therapeutic effect. Only methylprednisolone and the phenoxybenzamine and Ringer's combination appeared to alter acute T-2 toxicosis in a beneficial way. Due to the complexity of the pathophysiologic alterations which take place in acute T-2 toxicosis it is unlikely that one individual agent will be able to substantially improve survival following T-2 exposure, but rather a treatment protocol combining a variable number of agents will provide the most efficacy.

E. Studies on the Adsorptive Capacity of Activated Charcoals for T-2 Toxin and other Toxicants

Studies performed in our laboratory with activated charcoal have been directed toward development of an in vitro adsorption test that can be utilized to predict the efficacy of charcoal in preventing toxicoses in animals or humans. In order to validate the predictability of the in vitro

adsorption tests, we have tested three commercially available activated charcoals for their relative efficacy in preventing toxicoses in animals exposed to T-2 toxin and other model poisons. The other toxicants were chosen to represent a broad group of chemicals: chlorpyrifos, carbaryl, strychnine, and nitrite.

Our preliminary results indicate that a new "superactive" charcoal developed by Amoco Research Corporation and soon to be produced by Anderson Development Company has, except for nitrite, 2-3 times the efficacy against the various toxicants studied including T-2 toxin. This charcoal was very effective in preventing toxicoses in rats that had been given 2 times the oral $LD_{50}$ dose of T-2 toxin. Since our laboratory has found that T-2 toxin and its metabolites are excreted into the gastrointestinal tract following intravascular exposure (see Section IV), activated charcoal may be beneficial for treatment, as well as prevention, of trichothecene toxicoses, regardless of the route of exposure. A major advantage of activated charcoal is that it can be safely given to a conscious patient at home or in the field.

II. Subacute Toxicity and Immunotoxicity Following Inhalation and Dermal Exposure of Pigs to T-2 Toxin

Topical application of 0 and 15 mg/kg T-2 toxin in 0.75 ml DMSO was performed in swine. Representative animals were subsequently killed for pathologic examination on days 1, 3, 7 and 14. Grossly, the areas of skin exposed to T-2 toxin were swollen and progressively became dark red to dark purple. The affected skin became ulcerated after day 7 and was focally separated from the underlying tissue at the border of the exposure area on day 14. Histologically, the initial lesion began by degeneration of the stratum germinativum of the epidermis and mild neutrophilic infiltration of the upper dermis. The extent and severity of the changes then increased gradually with severe, necrotizing dermatitis apparent on days 7 and 14. Hyperkeratosis,

hyperplasia and fibroplasia began to appear on day 7 and became more prominent on day 14. The changes in the internal organs were minimal.

The immunotoxicity of dermally-applied T-2 toxin was evaluated in a group of 14 pigs which received the same treatment as described above and were bled on days -2, 1, 3, 5, 7, 10, 14, 20, 22, 24, 26, 28 and 31. It appeared that the purified peripheral blood lymphocytes of pigs exposed to T-2 toxin had significantly lower responses to the T-cell mitogens phytohemagglutinin and concanavalin A approximately 20 days after dosing, but the differences became nonsignificant by day 31. Significantly higher responses to the B-cell mitogen, lipopolysaccharide, were seen in the treated group during the first 10 days or so. No differences were found in anti-sheep red blood cell antibody titers between treated and control groups. Significant neutrophilia was seen in the T-2 toxin treated group during the first 2 weeks. The test group had significantly lower levels of serum albumin after one week of exposure, but significantly higher levels of serum globulin after 5 days of exposure. Serum alkaline phosphatase activities in the test group were significantly lower than those of the control group after day 3 of exposure. Blood glucose of the treated group was significantly lower during the first 2 weeks. A significant increase in the rectal temperatures of T-2 treated pigs occurred in the first 2 weeks. Significant differences between mean body weights of T-2 treated and control groups were noted after the first week of treatment.

III. Ultrastructural Study of T-2 Toxicosis in Swine

The effects of T-2 toxicosis, were evaluated in 8 pigs. They were administered T-2 toxin in intravenous doses of 0.0 mg/kg (2 pigs) and 0.6 mg/kg (6 pigs) dissolved in 2.5 ml of 50% ethanol and were killed 24 and 48 hours later. On gross examination, pancreatic edema, multifocal subendocardia 1 hemorrhages and pinpoint white foci were present scattered

throughout the myocardium of one pig killed at 48 hours. Myofiber degeneration and necrosis with contraction bands were seen in all pigs, mainly in the subendocardial region. Although the lesions were present throughout the heart, they were more predominant in the atria, papillary muscles of the left ventricle, and lower left and upper right ventricles. Multifocal myofiber necrosis with mineralization was noted in the pig with gross lesions. In addition, myofiber vacuolization was another morphological alteration observed in some affected muscle bundles. Vacuolization was more often detected in papillary muscles of the left ventricle. Ultrastructural changes consisted of areas of sarcoplasmic edema with myofibrillar disorganization and loss of Z and M bands, as well as glycogen accumulation in mildly affected myocytes. In severely damaged myocytes, hypercontraction bands with myofibrillar lysis or marked distension of sarcoplasmic reticulum with myofibrillar lysis was evident.

Pancreatic changes consisted of multifocal acinar degeneration and necrosis. These changes became a suppurative necrotizing pancreatitis in the pigs killed at 48 hours. Early ultrastructural changes consisted of rough endoplasmic reticulum dilation and disorganization as well as mitochondrial swelling and loss of cristae.

IV. Metabolism of T-2 Toxin

Two female swine were dosed intravascularly (iv) with one millicurie of tritiated T-2 toxin at a dosage of 0.15 mg/kg body weight. Both animals were sacrificed after 4 hours to follow tissue distribution patterns. The mean plasma elimination phase half-life was 90 minutes for T-2 and metabolites (total radioactivity) versus 13.8 minutes for the parent compound (T-2) alone, indicating that T-2 is rapidly metabolized and eliminated in swine. A significant portion of the dose, 18.2% and 46.8% was excreted in the urine of the two swine, respectively, within 4 hours after dosing. In addition, 14.1%

and 1.3% of the administered dose was present in the bile of the two swine 4 hours after dosing. The mean residue levels for T-2 and its metabolites in tissues were very low and never exceeded 65 ppb of total metabolites, with no individual metabolite exceeding 4 ppb.

A significant portion of the dose, 18.9% and 32.0%, was also detected in the GI tract, including contents, of each pig. The presence of radioactivity in the different sections of the GI tract corresponded to the lesions reported in iv-dosed swine. The ileum, which had the most severe lesions, contained the greatest amount of toxin (12% and 19% of the dose, respectively) and the duodenum, with no apparent lesion, the least (<0.3% of the dose). Less than 0.08% of the dose was detected in the feces. Identification of metabolites was accomplished by both one- and two-dimensional thin layer radiochromatography. The major metabolites identified were 3'-OH T-2, HT-2 and 3'OH HT-2 and their glucuronide conjugates in addition to T-2 glucuronides. Lesser amounts of neosolaniol, T-2 triol, 4-deacetylneosolaniol, T-2 tetraol and their conjugates were also present. The concentration of free T-2 was negligible in all samples following the initial distribution phase. Metabolite profiles varied greatly depending on the type of tissue or fluid sampled.

In vitro metabolism studies with T-2 toxin using swine and rat liver microsomes are in progress. The major metabolite produced in microsomal systems from both species was HT-2. Other metabolites produced include: 3'-OH T-2, neosolaniol, 3'-OH HT-2, T-2 triol, 4-deacetylneosolaniol and T-2 tetraol. In addition, several new compounds were detected which have not been identified. Results from these in vitro studies were instrumental in developing TLC radiochromatographic and gas chromatographic methods for analysis of metabolites from T-2 dosed swine.

## V. Residue Analysis/Methods Development

Methods for analysis of T-2 and metabolites in bile, plasma and urine were developed and refined. The method involves extraction with XAD-4 followed by cleanup on florisil and C18 cartridges. Final separation and quantitation of the heptafluorobutyryl derivatives was accomplished by capillary gas-liquid chromatography using electron capture detectors.

The method described was used to monitor metabolite profiles in swine topically administered T-2 toxin at 15.0 mg/kg bw (see Section II). No T-2 or metabolites were detected in the blood, urine or bile of these swine at 1, 3, 7 or 14 days post-dosing. T-2 and several metabolites, however, were detected in the skin and fat. The mean concentrations of T-2 toxin in the skin at the site of application on post-dosing days on 1, 3, 7 and 14 were 220, 247, 220 and 41 ppm respectively.

Blood and urine samples from swine administered T-2 toxin intravascularly at a dosage of 4.8 mg/kg were analyzed by capillary gas chromatography with electron capture detection. In most samples the major unconjugated metabolite detected was 3'-OH HT-2. Other metabolites identified were: T-2 tetraol, T-2 triol, 4-deacetylneosolaniol, HT-2, neosolaniol and 3'-OH T-2. In all samples of either plasma or urine, the concentration of the parent compound T-2 toxin was either nondetectable or was present in amounts approximately 100 times less than the predominant metabolite 3'-OH HT-2.

## VI. Trichothecene Production/Characterization

We have produced over 20 g of purified crystalline T-2 toxin in our laboratory for use in a variety of experiments. Current methods for production involve culturing *F. tricinctum* on vermiculite coated with glucose-yeast extract media. After incubation for 3 weeks the cultures are extracted with ethyl acetate and the T-2 toxin is purified by rapid column chromatography, HPLC and multiple recrystallization. In addition to

production of T-2 toxin, isolates of <u>Fusarium</u> have been collected and are being screened for production of a variety of trichothecenes. The screening method involves culturing the isolates on vermiculite media for 2 or 4 weeks. After extraction with acetonitrile, trichothecene profiles are monitored by thin layer and gas chromatography. Although this work is still in progress, preliminary data indicates several isolates have the capacity to produce zearalenone, deoxynivalenol or T-2 toxin.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I.  PATHOPHYSIOLOGY OF T-2 TOXICOSIS IN SWINE

A.  INTRAVASCULAR STUDIES--HEMODYNAMICS, VASOACTIVE MEDIATORS, HEMATOLOGY AND SERUM BIOCHEMISTRIES--Roseanne M. Lorenzana (see Appendix A, B)

OBJECTIVE

1.  To determine the clinical and physiologic changes resulting from intravascular exposure of T-2 toxin.

2.  To determine the nature of the acidosis in T-2 toxicosis.

INTRODUCTION

In swine given T-2 toxin intravascularly (0.6 and 2.4 mg/Kg), cardiac output and aortic mean pressure declined early, while heartrate increased. Increased concentrations of plasma norepinephrine, epinephrine, thromboxane $B_2$ and 6-keto-PGF$_{1\ alpha}$ were detected. Each individual animal demonstrated a significant increase or decrease in body temperature. There was a decrease in the partial pressure of arterial oxygen. However, in the low-dose group, when the partial pressure of arterial oxygen subsequently began to increase, the partial pressure of carbon dioxide demonstrated a tendency to decrease. Pulmonary vascular resistance increased, but systemic vascular resistance was reduced in the low-dose group, and no trend was detected in the high-dose group.

Increases in the serum concentrations of blood urea nitrogen, creatinine, phosphorus, and in the activities of aspartate amino transferase and alkaline phosphatase probably reflected secondary changes that occurred during the shock syndrome. Total serum calcium decreased along with a decrease in blood pH. The serum concentrations of magnesium and potassium increased only in the high-dose group. The pattern of the leukogram reflected a stress response. An early leukocytosos was followed by a leukopenia. Nucleated red blood cells were observed in blood smears from both toxin-dosed groups.

A major portion of this work was discussed in the First Annual progress report (March, 1983 pp.6-42). These data have been further analyzed to look for statistical correlations that could identify the mode of action of T-2 toxin. Analysis of the calculated values of total peripheral and pulmonary resistance have added important information to the understanding of the pathophysiology of T-2 toxicosis.

METHODS

Swine were used to characterize the pathophysiology of acute T-2 toxicosis. It is known that the cardiovascular and pulmonary physiology of swine is very similar to that of man, and swine are of sufficient size to allow collection of multiple blood specimens. In our experiment, purified crystalline T-2 toxin was dissolved in 6 1/2 ml of 70% ethanol and infused via the pulmonary artery. White, crossbred female swine ranging from 40 to 60 kilograms were purchased from a local source and allowed to acclimate to our facility prior to surgery. Catheters were surgically placed in the pulmonary artery, left atrium and aorta. The catheter tips were buried in the subcutis of the dorsum and the pigs were allowed to recover for 3 weeks. On the day of the study, each pig was restrained in a webbed stanchion, the skin over the catheter tips was anesthetized with lidocaine, small skin incisions were made and the catheters were attached to sampling and measuring devices. The animals rested comfortably in this arrangement and were conscious throughout the experiment. Pressures in the left atrium, pulmonary artery, and aorta were recorded by a Physiograph[R] (Gilson Medical Electronics, Inc., Middleton, WI), cardiac output was determined by a dye-dilution technique and blood samples for clinical chemistries were withdrawn from the aortic catheter. There were three groups of animals in the formal study: High = 4.8 mg/kg, Low = 0.6 mg/kg, and vehicle = 70% ethanol.

## STATISTICAL EVALUATION

Widely differing predose values and postdose variances in these data negated standard analyses of variance methods. As an alternative approach, each animal in the three (High, Low and Control) groups was tested for significant upward or downward trends in each parameter, these trends being assessed for significance by both Pearson's and Kendall's tau two-tailed correlation analyses, with the time sequence of successive observations serving as the independent variate. In this approach, all animals were individually tested against the hypothesis that treatment had no nonrandom effects upon the monitored parameters. Intergroup differences were then judged by comparing the sets of intragroup tests with each other. In this way, for example, five high-dose animals, all showing a significant increase in a particular parameter, were clearly behaving differently than a population of five low-dose animals all showing either no change or the opposite change in the same parameter. A value of P less than 0.05 was considered statistically significant. During the early time period when the high-dose animals were alive, these intergroup comparisons were buttressed by the Kruskal-Wallis nonparametric one-way analysis of variance (Ghent, 1974; Nie et al., 1975).

## RESULTS AND DISCUSSION

Clinical signs were readily apparent in swine given intravascular T-2 toxin. Both the high and low-dose animals reacted similarly in the first several hours. They began to chew and salivate within fifteen to thirty minutes. This was followed by persistent vomition and, in some animals, watery diarrhea and flatulence. All the swine were notably restless. Within the first hour most animals exhibited abdominal straining associated with prolonged exhalation, and purplish mucous membranes began to be apparent.

Urine output was negligible at two hours after administration of T-2 toxin at the high dose, while swine in the low dose and vehicle groups continued to produce urine. The skin was noticeably reddened, and the ears and limbs were cold to the touch by two to three hours. After three to four hours, the animals appeared drowsy but could be aroused.

Swine in the high-dose group died as early as five hours after toxin administration. In contrast, the signs in the animals of the low-dose group began to regress between five and six hours and at twelve hours, the swine appeared clinically normal except for distinct reductions in alertness and activity. The alterations in body temperature were unusual. Body temperature was stable in control swine, but in the high-dose group, every animal's temperature either increased or decreased significantly. In the low-dose group, there was an early decrease in body temperature occurring between the time of dosing and 1 hour, thereafter.

Both cardiac output (CC) and aortic mean pressure (AOM) are considered in the calculation of total peripheral resistance (TPR) by the formula:

$$TPR = AOM/CO$$

Although heart rate eventually increased, both AOM and CO rapidly declined. In the high-dose group between predosing and 4 hours, the rates of decline in CO and AOM were not statistically different from one another. This resulted in an apparent lack of trend in the calculated value of systemic resistance (TPR). However, in other work from our laboratory in which swine received T-2 toxin, intravascularly at ~.8 mg/Kg, and were infused with radiolabelled microspheres, decreased perfusion occurred in some visceral organs. These data, in addition to our clinical observations of skin and limb perfusion, lead to the firm conclusion that peripheral vascular flow was, in fact, altered (See section IB). The calculated resistance value probably reflects the resistance to flow through the limited circuit of the heart and lungs. The

absence of any trend in TPR in the high-dose group suggests that the closure of the peripheral capillary vasculature was sudden, intense and occurred quite early. The rates of decline of CO in the high and low-dose groups were not statistically different, suggesting that the cardiac effects were similar in both T-2 dosed groups. However, the microcirculation retained responsiveness in the low-dose group and following a reduction in AOM and TPR, the vasculature responded to increase systemic pressure to bring the TPR back toward the predosing value.

The rise in the concentration of thromboxane $B_2$ ($TXB_2$) compared well with the rise in pulmonary vascular resistance (PVR). PVR increased substantially after 2-3 hours, and remained elevated after 4 hours, whereas $TXB_2$ concentrations were declining by this time in both groups. In addition to thromboxane, acidosis and elevated catecholamine concentrations may have contributed to the increase in pulmonary vascular resistance. The early decrease in arterial partial pressure of oxygen ($PaO_2$) in both groups may indicate that the source of the elevated PVR was primarily arteriolar. The subsequent increase in $PaO_2$ in the low-dose group in the face of continued elevation of PVR may indicated a shift to primarily venule constriction. A metabolic acidosis was characterized by a decline in blood pH, accompanied by an increase in the plasma concentration of lactic acid, but not uric acid, and the absence of any substantial decrease in $pCO_2$, suggesting minimal respiratory compensation.

In summary, there were early and simultaneous reductions in CO and AOM, and increases in 6-keto-PGF$_{1,alpha}$ plasma catecholamine concentrations and heart rate. These were accompanied by a decrease in TPR in the low-dose group but no change in TPR in the high-dose group. In both T-2 treated groups, an

increase in PVR occurred along with increases in norepinephrine, $TXB_2$ and acidemia.

The action of T-2 toxin apparently did not permit normal cardiovascular responses to the presence of elevated catecholamines. The effects could be attributable to primary cardiac and both primary and secondary vascular factors.

Another hypothesis may also be considered. Factors which could be possibly mediated by nervous system dysfunction in this toxic syndrome include: the sudden decrease in CO and AOM, the differing responses of the microcirculation in the high and low-dose groups, the delayed increase in heart rate, the erratic secretion of catecholamines in the high-dose group, and in the low-dose group, the sustained norepinephrine concentrations in the face of decreased AOM in the low-dose group, the presence of cholinergic clinical signs, signs suggestive of mental depression, the lack of appropriate respiratory compensation for the severe metabolic acidosis and altered thermoregulation. Further studies should be performed to determine whether a theory of nervous system mediation of T-2 toxicosis would be substantiated.

Alterations in serum biochemical parameters were a result of the T-2 toxin-induced shock. Total calcium concentration decreased significantly in both high and low-dose groups. It was, however, the reduction of albumin bound calcium that was responsible for the overall decrease. A probable initiating impetus for the decline in bound calcium was acidic blood pH. Hydrogen ions competitively displace bound calcium from albumin making it unbound (ionic). Homeostatic mechanisms work to maintain a constant concentration of unbound calcium. This occurs within minutes, and the result is a reduction in bound calcium. Subsequent elevations in phosphorus and magnesium may have exerted additional hypocalcemic effects.

The pattern of the leukogram of animals that received T-2 toxin reflected a stress response. The initial leukocytosis followed by leukopenia resembled an epinephrine response. In the low-dose group, the neutrophilia, lymphopenia, and eosinopenia at 24 hours postdosing were compatible with the effects of endogenous steroids in response to stress.

The appearance of circulating nucleated red blood cells without a concommitant decrease in numbers of mature red blood cells is suggestive of injury to the bone marrow endothelium. Since no nucleated red blood cells were observed in the low-dose group at 24 hours, it may follow that the damaging influence, perhaps ischemia or a cytotoxic effect of T-2 toxin and/or its metabolites, was resolved.

Copies of the manuscripts submitted for publication to Fundamental and Applied Toxicology are included in this report (Appendix A and B) .The data, accompanied by complete discussions, are included in the manuscripts. Portions of this work were presented by Dr. Lorenzana at the annual meeting of the Federation of the American Society of Experimental Biologists (FASEB) in April 1984 and at the FASEB Research conference, "Diagnosis, Toxicity and Therapy of Tricothecene Mycotoxicosis", in June, 1984.

B. SYSTEMIC DISTRIBUTION OF BLOOD FLOW DURING T-2 TOXICOSIS—Gregg R. Lundeen

OBJECTIVE

To determine how the total body distribution of cardiac output is altered as acute T-2 toxicosis progresses over time.

HYPOTHESIS TO BE TESTED

The fraction of cardiac output supplying the viscera, musculature, skin and kidneys is vastly reduced in T-2 toxicosis as the cardiac output declines and the animal conserves blood flow to the brain and heart.

INTRODUCTION

The effect of the shock syndrome associated with T-2 toxicosis upon the systemic distribution of blood flow in swine has not been previously reported. The use of radionuclide-labeled microspheres allows determination of cardiac output as well as distribution of blood flow to the various organs. We are, therefore, employing this technique to examine the cardiovascular effects of T-2 toxin in pigs at 0.6 mg/kg and 2.4 mg/kg and comparing them to predosing and to vehicle control values. Swine are considered to be an appropriate animal model because of anatomic and physiologic similarities to humans with respect to the cardiovascular, digestive and integumentary systems, nutritional requirements and mineral metabolism.

METHODS

Prior to beginning the study, fluid-filled catheters were implanted in the main pulmonary artery and left atrium of each animal. The ascending and descending aorta were also catheterized via the left internal thoracic artery and right femoral artery, respectively. All catheters were heparinized and directed subcutaneously near the center of the dorsum. The swine were allowed several weeks to recover from the surgery.

On the day of the study each pig was restrained by suspension in a sling such that the extremities hung freely below. After infiltration of the skin

with lidocaine hydrochloride the previously implanted catheters were exteriorized via small skin incisions. Heart rate and arterial blood pressure were monitored at 15 minute intervals for at least one hour prior to beginning organ blood flow determination to ensure that a hemodynamic steady-state preceded the study.

The following measurements were made on each animal: (1) arterial and mixed venous blood-gas tensions, (2) pressures in the aorta, pulmonary artery and left atrium, (3) cardiac output by the dye-dilution technique, and (4) cardiac output and organ blood flow using the microsphere technique.

Organ blood flow was determined by left atrial injection of an ultrasonicated, well-agitated suspension of 15 um diameter radionuclide labeled microspheres. Five different isotopes were used in each animal. For each blood flow determination, 4-5 million microspheres were injected. Beginning just prior to the injection of microspheres and continuing or 90 seconds post-injection, reference arterial blood was withdrawn from the abdominal aorta at a constant rate. This procedure was carried out with careful hemodynamic monitoring, and all criteria for organ blood flow and cardiac output determination by the microsphere method were satisfied (Archie et al., Buckberg et al.). Adequate mixing of microspheres was demonstrated by similar values of blood flow obtained for members of paired organs (i.e., cerebral hemispheres, adrenal glands and kidneys).

Upon completion of the experiment, the animals were anesthetized with an intravenous injection of sodium pentothal and killed by exsanguination. The organs for which we wished to measure blood flow were removed and weighed. Each organ was individually minced and placed into pre-weighed vials which were then weighed again. Tissue heights from the bottom of the vials did not exceed 1 cm. The vials were then counted using a gamma well scintillation counter connected to a multichannel analyzer with appropriately selected

energy windows. Raw counts from the tissues were corrected for background and crossover, and compared with reference arterial blood to determine blood flow.

The swine were divided into 3 treatment groups: (1) vehicle control (70% ethanol), (2) low dose (0.6 mg/kg T-2 toxin) and (3) high dose (2.4 mg/kg T-2 toxin). Blood flow and hemodynamic measurements were then determined for each animal, as described above, at the following time intervals: (a) 0 hours (pre-injection), (b) 1 1/2 hours (post-injection), (c) 3 hours (post-injection), (d) 4 1/2 hours (post-injection) and (e) 6 hours (post-injection).

This study is still in progress. The values presented here are the means of only 2 or 3 animals (i.e. vehicle control n=3, low dose n=2, high dose n=2), therefore, the results should not be construed as being conclusive, but rather they are a representation of the trends that appear to be occurring during T-2 toxicosis. It should also be noted that one of the high dose animals died before readings were obtained for the 6-hour postinjection time period. Because of this, the values for the high-dose at that time, represents only one animal.

RESULTS AND DISCUSSION

Arterial blood-gas variables are depicted in Figure IB1. These findings correlate with our previous findings. In both T-2 treated groups, there were reductions in arterial blood pH and arterial carbon dioxide tension indicating the presence of a metabolic acidosis. No reduction in arterial carbon dioxide tension was present, however, in the low-dose group at 1 1/2 and 3 hours postdosing. Packed cell volume (Fig. IB2) was increased in both T-2 dosed groups which is similar to our previous findings (Lorenzana et al., Appendix A and B).

Following T-2 administration cardiac output Fig. IB3) was decreased. This trend appeared to reverse after 3 hours in the low-dose group, whereas in the

high-dose group cardiac output continued to decline. The decline in aortic mean pressure (AOM; Fig. IB4) tended to parallel the reduction observed in cardiac output, with the exception that the decrease in AOM in the high-dose group did not occur until after the 1 1/2 hour post-injection reading. Pulmonary artery mean pressure (PAM) is also presented in Figure IB4. The increase seen in the pulmonary artery mean pressure in the high-dose group would seem to indicate the presence of pulmonary vasoconstricton, which again is consistent with our previous findings. This effect was not apparent in the low-dose or the vehicle control groups.

Heart rate (Table IB1), which remained steady in the vehicle control group, was increased in both T-2 treated groups, apparently as a compensatory response to the drop in blood pressure. The decrease in stroke volume [computed as the quotient of cardiac output ($ml \times min^{-1} \times kg^{-1}$) and heart rate (beats $\times min^{-1}$)] (Table IB1) of the T-2 treated animals paralleled the reduction in cardiac output. Total peripheral resistance (Table IB1) was slightly decreased in the low-dose group while in the high-dose group it was increased. This latter finding is more in line with what would be expected from vasoconstrictive responses to the shock syndrome induced by T-2 toxin. The difference between this pattern of responses and that reported by Lorenzana, et. al. (Appendix A), is probably attributable to a 2-fold greater dosage of T-2 toxin in that study. Pulmonary vascular resistance (Table IB1) increased in both T-2 groups. This finding would appear to correlate with the aforementioned increase in pulmonary artery pressure.

Ventricular work was calculated as: cardiac output ($ml \times min^{-1} \times kg^{-1}$) $\times$ mean pressure (aortic or pulmonary arterial) $\times 1.36 \times 10^{-2}$ where 1.36 is the conversion factor for changing pressure in mmHg to $g/cm^2$. Left and right

ventricular work (Table IB1) was steadily decreased over time in both groups of T-2 treated animals which is primarily a result of the reduction in cardiac output.

Blood flow to the brain (Fig. IB5) decreased following T-2 toxin administration, particularly in animals dosed at 2.4 mg/kg. This effect is most likely due to vasoconstriction and a concomitant reduction in perfusion pressure. Blood flow to the brain in the low dose group fluctuated markedly, but the reason for this finding is not yet clear.

Blood flow to the myocardium (Fig. IB6) of swine dosed with 0.6 mg/kg T-2 toxin fluctuated in a manner similar to that seen in brain blood flow. The animals dosed with T-2 toxin at 2.4 mg/kg exhibited a sustained reduction in blood flow that paralleled the reduction in arterial pressure.

Adrenal blood flow (Fig. IB7) was notably increased in both low and high-dose groups following T-2 toxin administration. This correlates well with our previous findings that show a significant rise in circulating catecholamines in swine dosed with T-2 toxin. The increased blood flow to the adrenals was maintained throughout the time course of the experiment.

Renal blood flow (Fig. IB8) was decreased below control values following T-2 administration. This decrease in renal blood flow in conjunction with the drop in aortic mean pressure may account for the cessation of urine production that occurs in animals dosed with T-2 toxin.

In both groups of swine dosed with T-2 toxin there was a drastic reduction in blood flow to the spleen (Fig. IB9). The intense splenic contraction may be the result of increased levels of circulating catecholamines which stimulate splenic smooth muscle. Splenic contraction also supports the finding of increased hematocrit brought about by a dumping of red blood cells into the general circulation. However, the local damaging effects of T-2

toxin on lymphoid tissue may also result in compromised circulation in this T-2 sensitive organ.

Hepatic arterial blood flow (Fig. IB10) was increased following T-2 administration and the increase was maintained despite the decrease in arterial pressure. The exact reason for this increase is unknown, but it may be mediated locally and may influence the clearance of T-2 toxin and metabolites.

Stomach blood flow (Fig. IB11) was decreased at a rate which somewhat paralleled the decrease in aortic pressure. In small intestine and large intestine, however, blood flow was maintained near or above predosing values in spite of the shock syndrome (Fig. IB12, 13). The reason for this finding is not understood, but blood flow may be increased to the gut in response to the severe local cytotoxic and secondary reflexive responses that result in diarrhea in animals dosed with T-2 toxin.

FUTURE STUDIES:

Blood flow studies in intravascularly dosed swine are continuing this summer and fall (1984). It is planned to begin blood flow studies in swine dosed with T-2 toxin via the inhalation route of exposure in January 1985, so as to allow completion by March 31, 1985.

REFERENCES

Archie J P, Fixler D E, Ullyot D J, Hoffman J I E, Utley J, Carlson E: Measurement of cardiac output with and organ trapping of radioactive microspheres. J Appl Physiol 35:148-154, 1973.

Buckberg G D, Luck J C, Payne D B, Hoffman J I E, Archie J P, Fixler D E. Some sources of error in measuring regional blood flow with radioactive microspheres. J Appl Physiol 31:598-604, 1971.

Table IB1: Hemodynamic Variables.

| | VEHICLE (n=1) | | | | | 0.6 mg/kg (n=2) | | | | | 2.4 mg/kg (n=2) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (Hours Post Dosing) | | | | | (Hours Post Dosing) | | | | | (Hours Post Dosing) | | | | |
| | 0 | 1 | 2 | 4 | 6 | 0 | 1 | 2 | 4 | 6 | 0 | 1 | 2 | 4 | 6 |
| Heart rate (beat·min⁻¹) | 102 | 108 | 109 | 95 | 102 | 135 | 108 | 180 | 177 | 202 | 116 | 119 | 176 | 186 | 190 |
| Stroke Volume (ml·beat⁻¹·kg⁻¹) | 1.70 | 1.70 | 1.55 | 1.40 | 1.75 | 1.50 | 1.34 | 0.64 | 0.70 | 0.74 | 1.46 | 1.08 | 0.50 | 0.33 | 0.52 |
| Total Peripheral Resistance (mmHg·ml·min⁻¹·kg⁻¹) | 0.727 | 0.747 | 0.906 | 0.814 | 0.738 | 0.600 | 0.646 | 0.624 | 0.452 | 0.447 | 0.743 | 1.055 | 0.447 | 0.954 | 0.485 |
| Pulmonary Vascular Resistance (mmHg·ml·min⁻¹·kg⁻¹) | 0.093 | 0.099 | 0.120 | 0.113 | 0.106 | 0.110 | 0.134 | 0.186 | 0.162 | 0.143 | 0.145 | 0.240 | 0.378 | 0.610 | 0.121 |
| Left Ventricular Work (kg·mm·min⁻¹·kg⁻¹) | 298 | 285 | 267 | 275 | 305 | 332 | 176 | 98 | 87 | 114 | 287 | 218 | 88 | 41 | 65 |
| Right Ventricular Work (kg·mm·min⁻¹·kg⁻¹) | 47 | 45 | 45 | 49 | 54 | 74 | 44 | 30 | 32 | 41 | 58 | 43 | 30 | 12 | 16 |
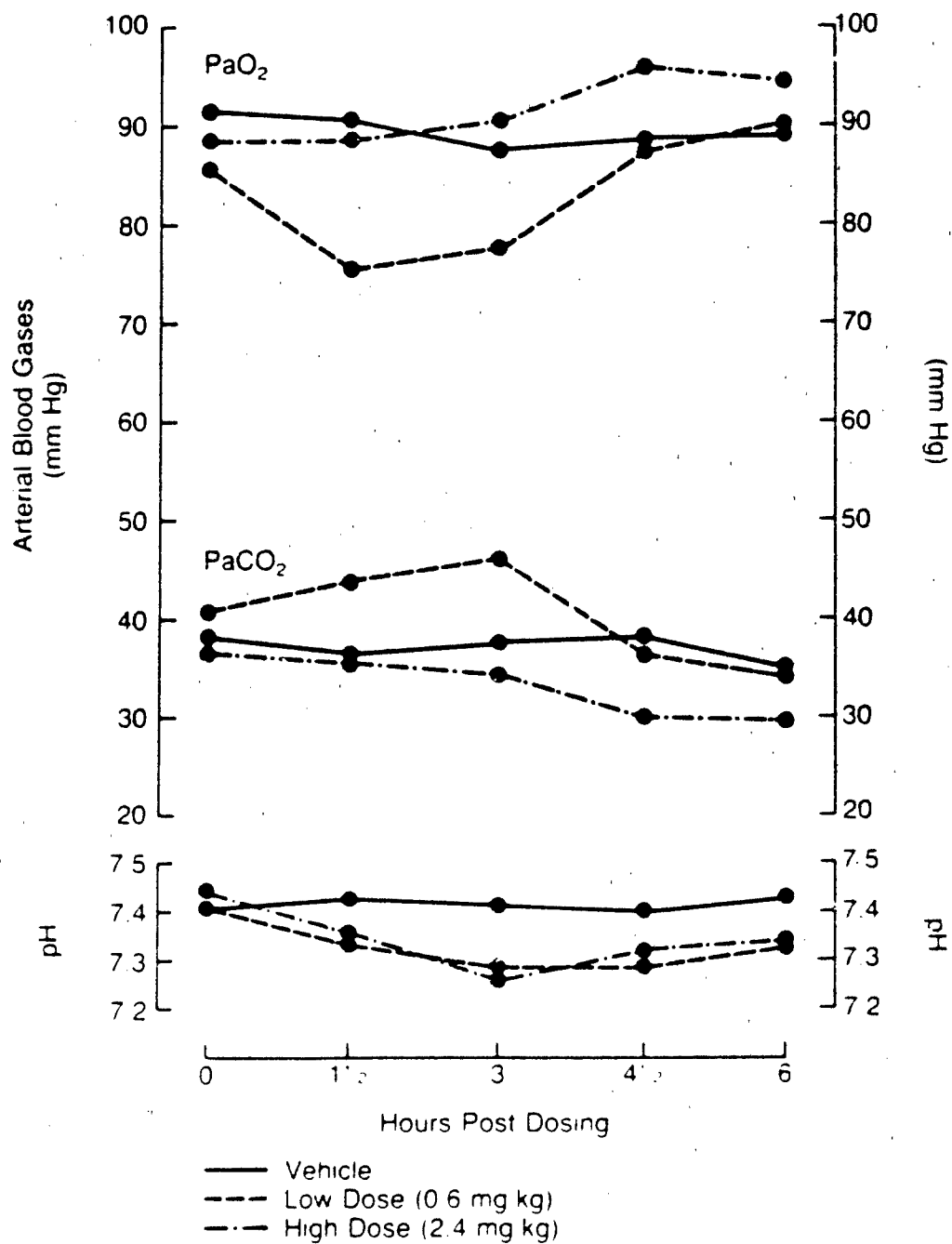
Fig. IB1 Arterial blood-gas tensions and pH.
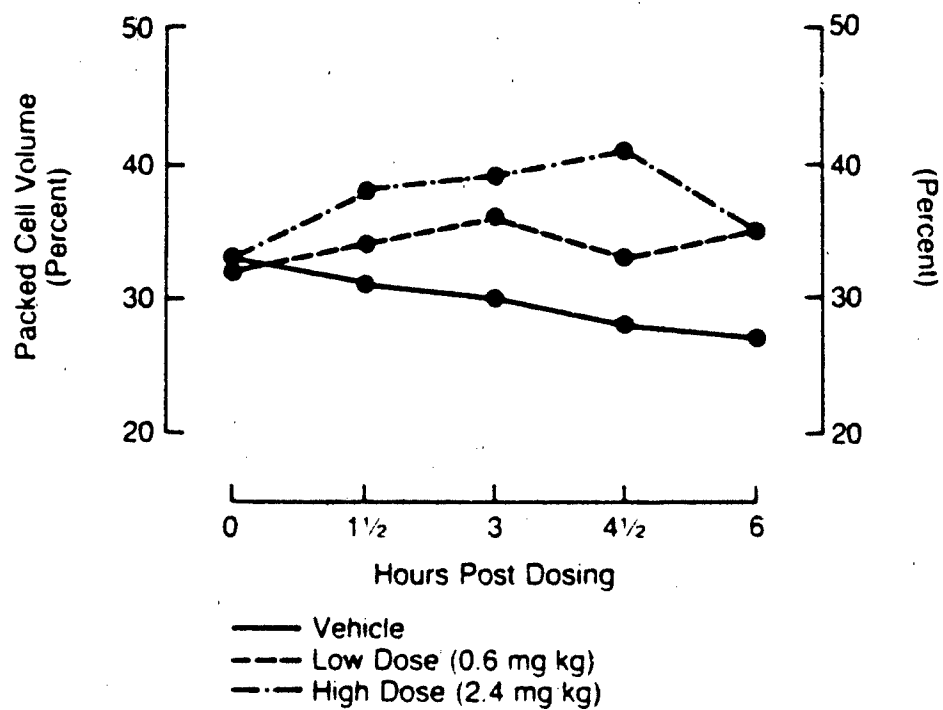
Fig. IB2  Arterial packed cell volume.  Note the increase in hematocrit
          following T-2 administration.
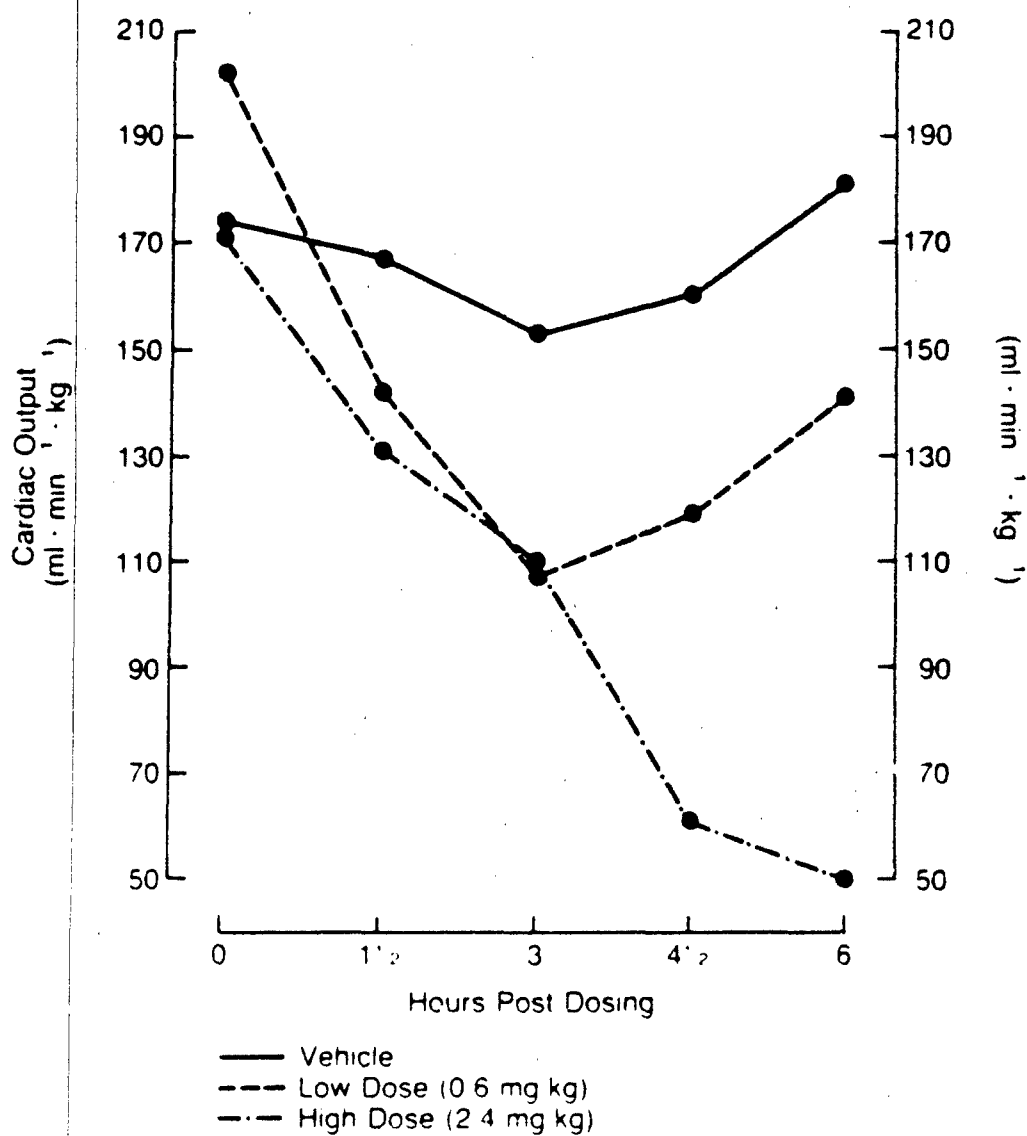
Fig. IB3  Cardiac output in swine dosed with 70% ethanol, 0.6 mg/kg T-2 toxin,
and 2.4 mg/kg T-2 toxin.  Note the apparent recovery of cardiac out-
put in the low-dose animals while the high-dosed animals show a
continued decline.
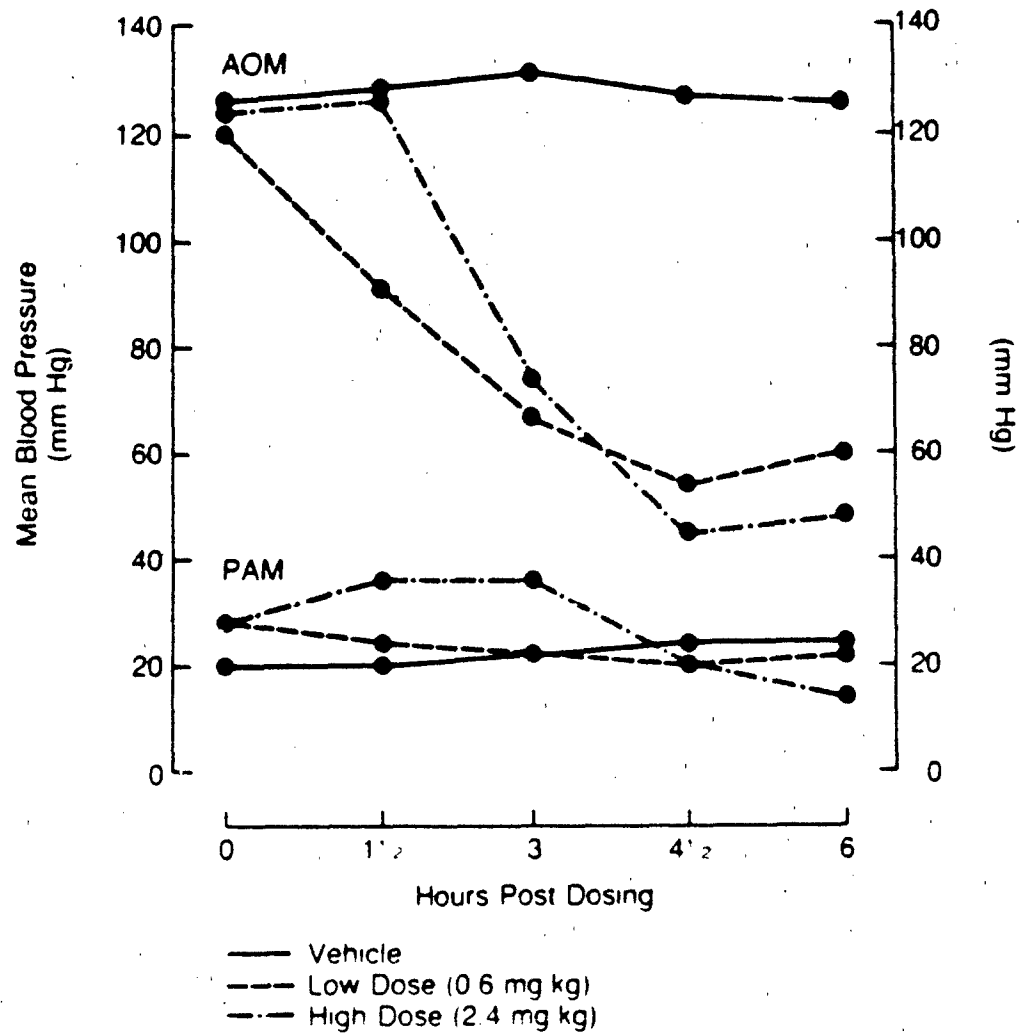
Fig. IB4  Mean aortic and pulmonary artery pressures.  Note the increase in
mean pulmonary artery pressure of the high-dose group.
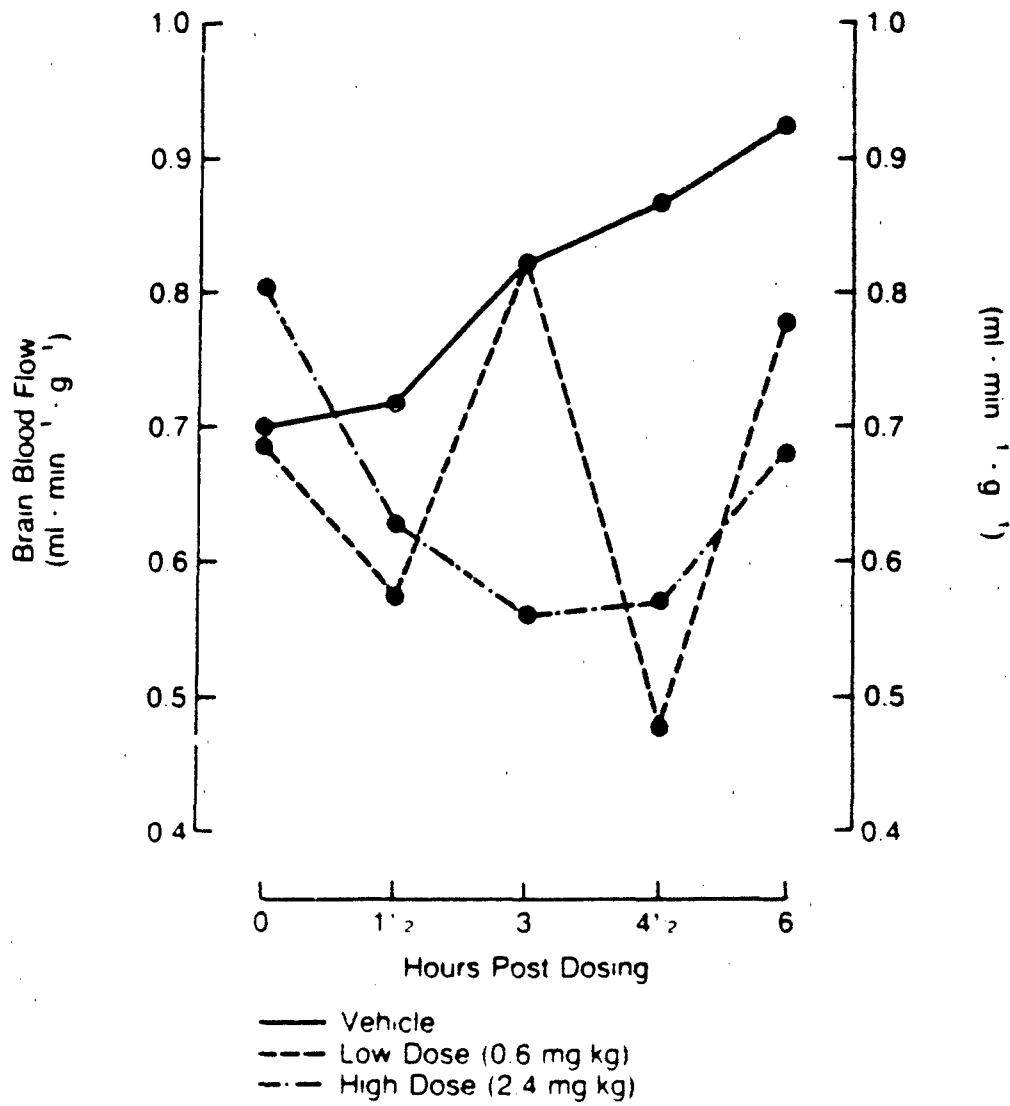
Fig. IB5 Alterations in blood flow to the brain of swine dosed with T-2 toxin.
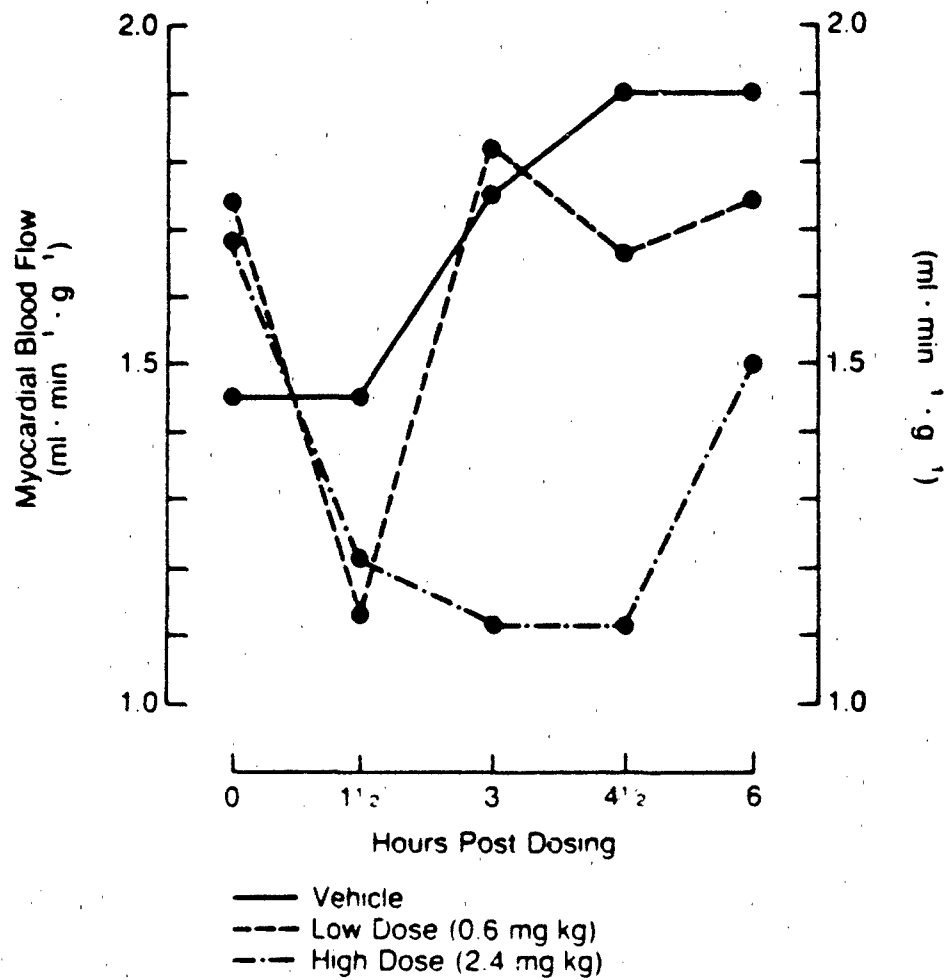
Fig. D36   Changes in blood flow to the myocardium of swine following the administration of 70% ethanol, 0.6 mg/kg T-2 toxin and 2.4 mg/kg T-2 toxin.
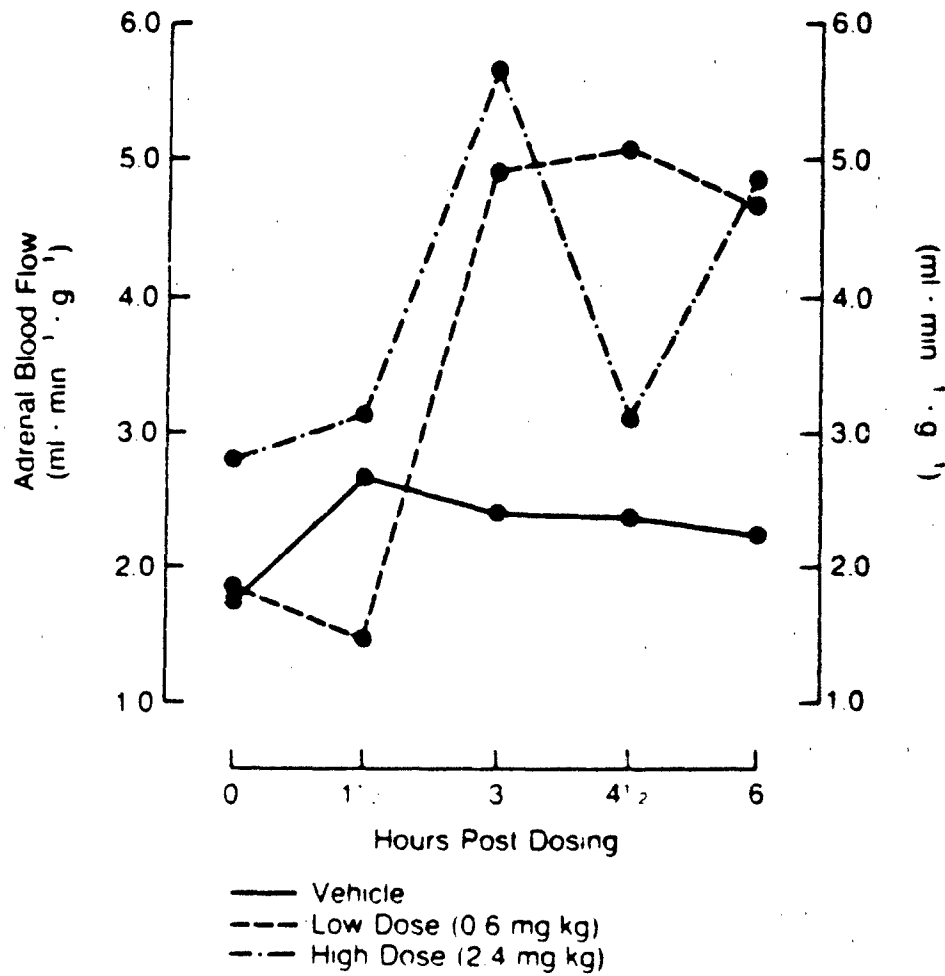
Fig. IB7  Blood flow to the adrenal glands of pigs after being dosed with T-2 toxin.  Note the increase in blood flow of both T-2 dosed groups.
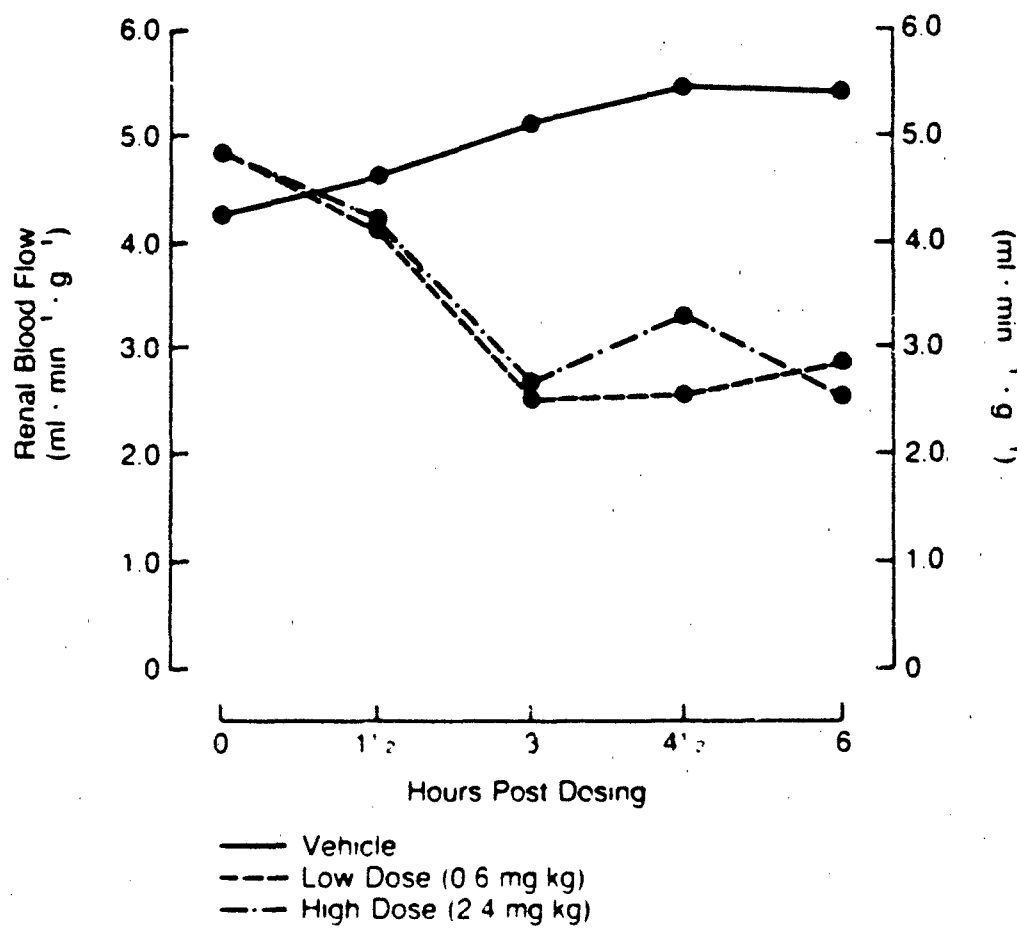
Fig. IB8 Renal blood flow in swine dosed with 70% ethanol, 0.6 mg/kg T-2 toxin and 2.4 mg/kg T-2 toxin.
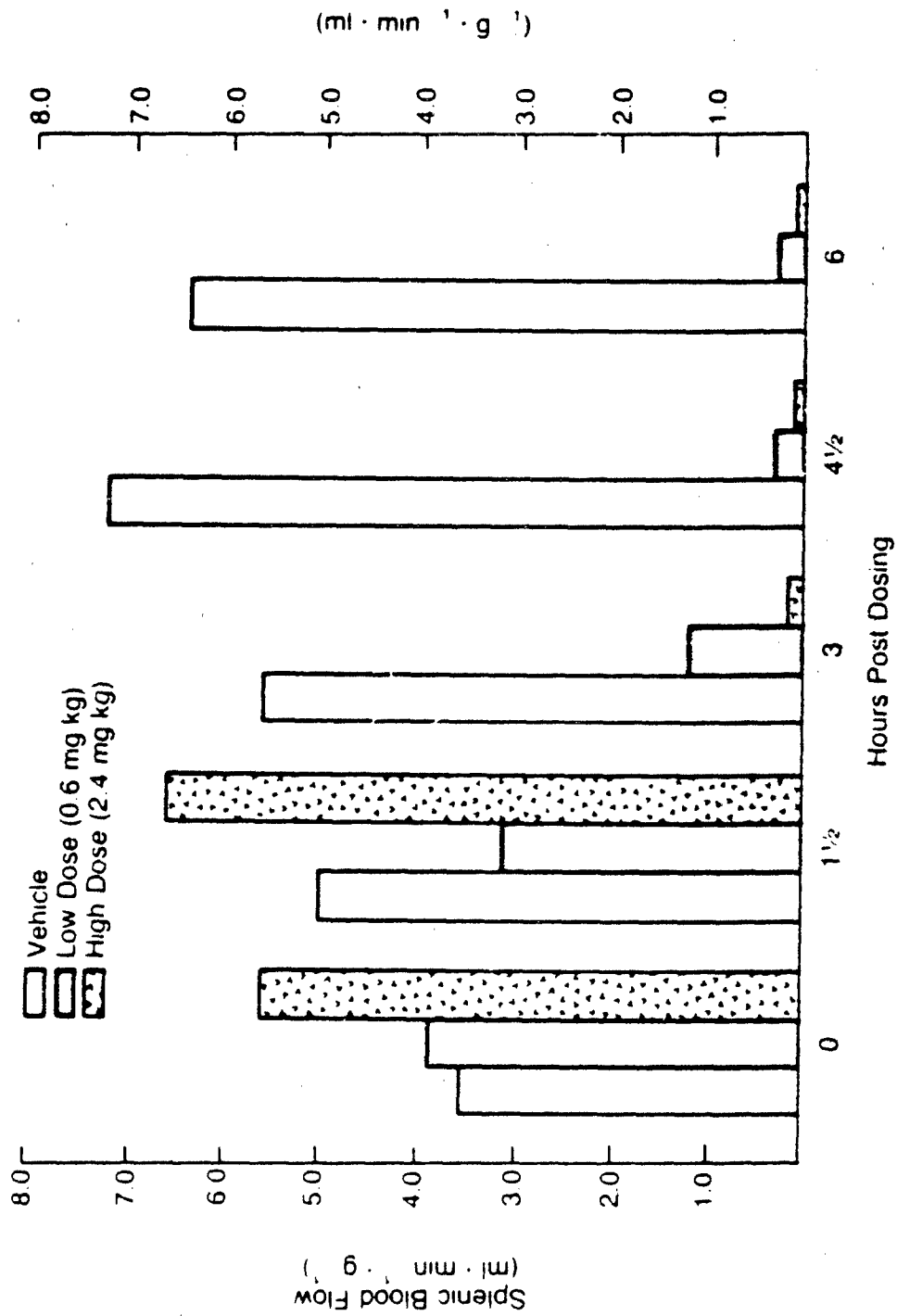
Fig. IB9   Alterations in blood flow to the spleen.  Note the tremendous decrease in
splenic blood flow of both T-2 dosed groups.
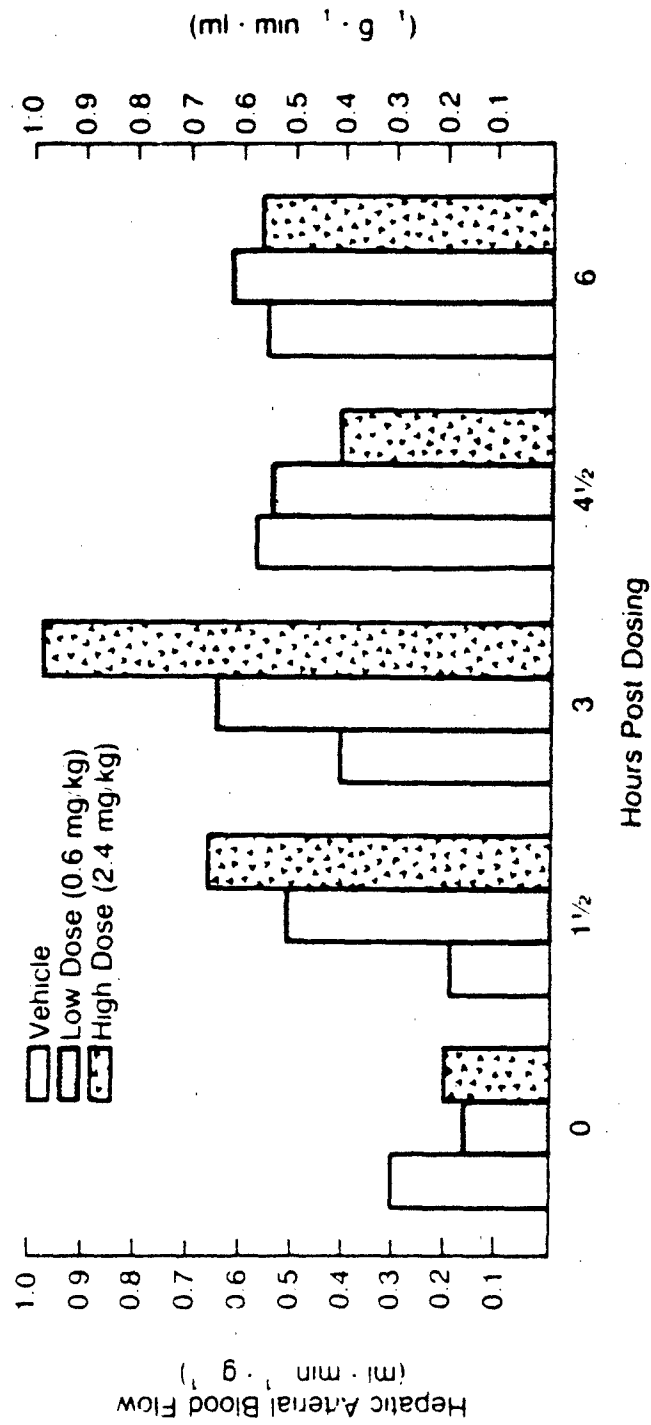
Fig. IB10  Hepatic arterial blood flow.
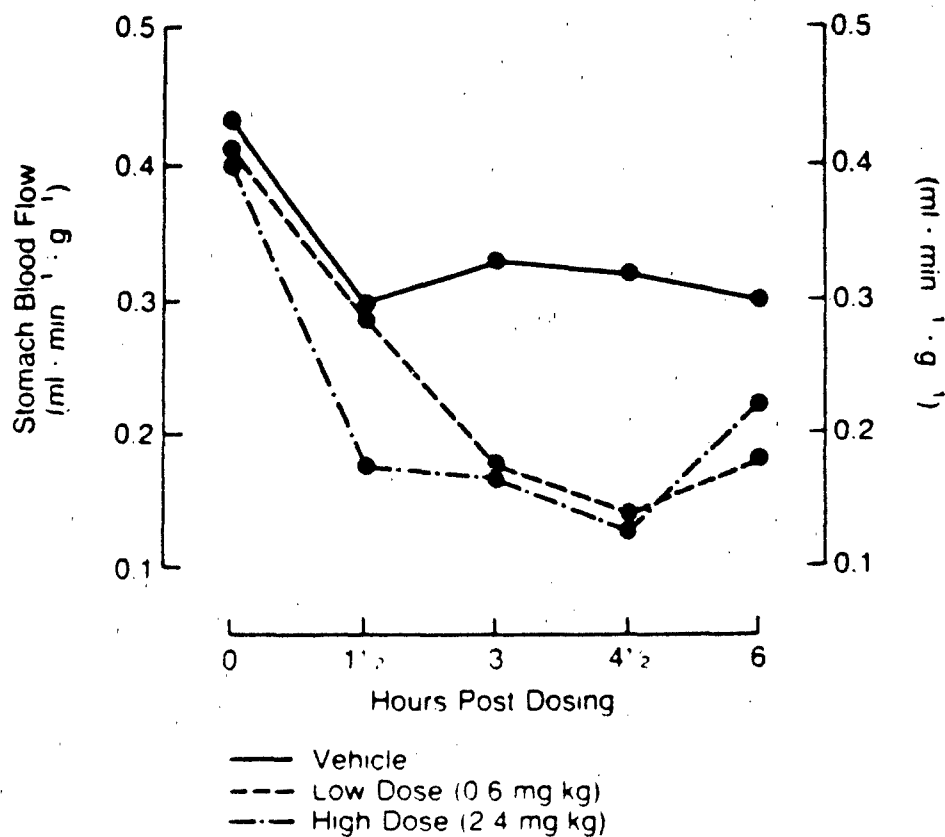
Fig. IB11 Changes in blood flow to the stomach brought about by T-2 tox_
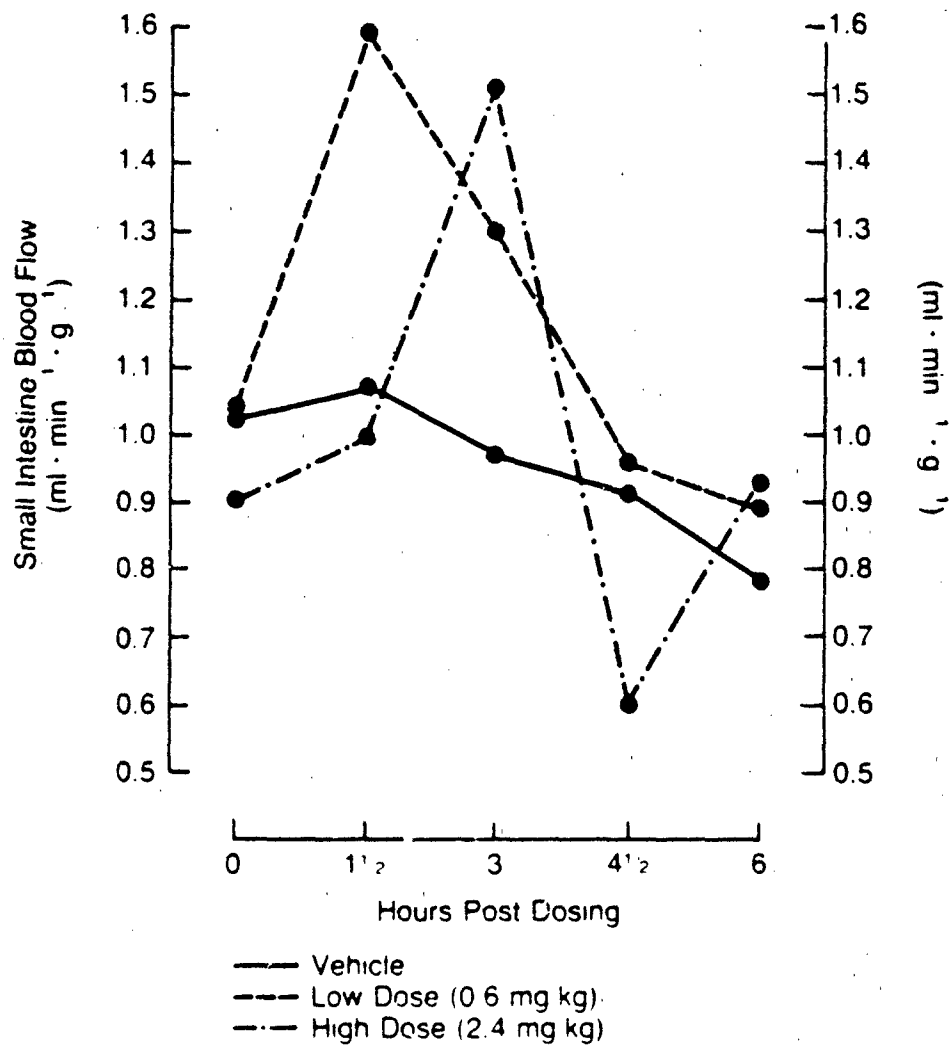
Fig. IB12 Small intestine blood flow in swine dosed with 70% ethanol,
0.6 mg/Kg T-2 toxin and 2.4 mg/Kg T-2 toxin.
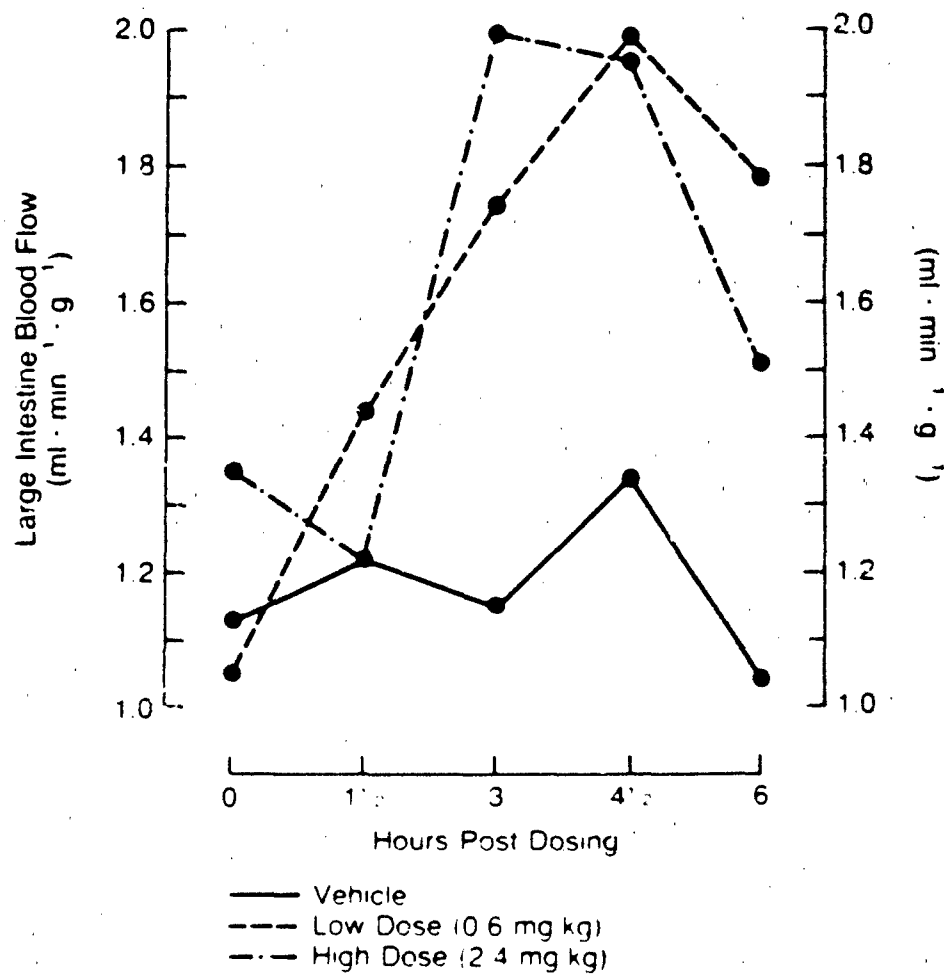
Fig. IB13  Alterations in blood flow to the large intestine induced by T-2 toxin. Note the elevated blood flow in both groups dosed with T-2 toxin.

C. DEVELOPMENT OF A CLOSED-SYSTEM INHALATION PROCEDURE FOR STUDYING DOSE-RESPONSE EFFECTS OF T-2 AEROSOL EXPOSURES--Richard J. Lambert

OBJECTIVES

1. To develop an inhalation exposure system and the techniques necessary to administer a lethal dose of T-2 toxin to swine.

2. To develop and validate a technique for determining the amount of T-2 toxin retained by the pig during exposure to the aerosol.

3. To compare local and systemic effects of T-2 toxicosis resulting from both inhalation and intravascular dosing.

INTRODUCTION

The pathophysiologic effects of inhaled T-2 toxin are not fully characterized nor understood. Our goal is to determine and describe the pathologic, physiologic, and immunologic effects of inhaled T-2 toxin using swine as an animal model for human exposure.

In order to reach that goal, it has been necessary to develop a system for administration of the toxin by inhalation. Due to the limited availability and high cost of T-2 toxin, the most efficient way to study the effects of inhaled quantities in an animal as large as swine is by direct delivery of the aerosol to the trachea. Although swine have been placed in enclosed chambers to study the effects of breathing air containing bacteria and dusts, information on methods for intratracheal administration of toxins to non-anesthetized pigs is lacking. As a result, it has been necessary to do the following:

1) Develop a non-chemical means of restraint for the pigs during the inhalation exposure period when they are isolated in a chamber. (The apparatus must not induce the pig to struggle yet it must be confining enough to limit the pig's movements);

2) Perfect a technique for securing the endotracheal tube and associated tubing so that they are not dislodged or separated during periods when the pigs struggle;

3) Find a means of preventing the endotracheal tube from becoming plugged with mucous during the administration of the T-2 aerosol;

4) Establish the equipment parameters that will result in the production of dry T-2 particles of a mass median aerodynamic diameter that is small enough to allow penetration of the toxin to the alveolar level in the lungs. Once these problems are solved, it is possible to administer the toxin and concentrate on its effects.

An equally important aspect of this project is the development of a method of quantitating the amount of T-2 toxin retained by the swine. In order to compare effects caused by inhaling the toxin with effects resulting from other means of administration, it is necessary to know the relative amounts retained by the different routes. Unlike the comparative ease of quantifying an injection of toxin, the accurate quantitation of the toxin retained as a result of inhalation exposure presents many unique problems.

The delivered dose can be determined by a number of methods, but many of them are relatively crude. Direct assay of the exposed tissues for T-2 toxin can be done. Most of the inhalation-exposed pigs in this study will be monitored for a number of hours after exposure. The rapid degradation and short half-life of T-2 toxin in the body as well as the massive amount of tissue preparation and analytical work that would be required precludes the use of this technique. Another method is to measure the duration of exposure, and air concentration of the test material, and then calculate exposure using assumed values for the animal's respiratory minute volume and percentage of retained aerosol (Cuddihy and Boecker, 1973). The alterations in an unanesthetized animal's respiratory pattern during aerosol delivery, and the

difficulties in estimating the percentage of retained aerosol make this a less than ideal method of determining dosages (Cuddihy and Boecker, 1973). A third method involves subtracting the amount of material recovered in the generating and collecting systems from the starting quantity. The amount remaining is assumed to have been received by the animal (Brain and Valberg, 1979). Quantitation of the material can be done by standard analytical techniques (gas chromatography for T-2 toxin), or by methods using radiolabelled T-2 or a concurrently administered isotope as a marker. When radioactive materials are used, the body burden can also be measured directly. The use of radioactive materials in dose determinations is a widely practiced and established technique (Cuddihy and Boecker, 1973; Brain and Valberg, 1979).

PRELIMINARY AND PREPARATORY WORK

A comfortable form of animal restraint was developed by modifying the moveable sling support system which has been used in the other pathophysiology studies. With this device, the pigs are supported under the thorax and abdomen with their legs hanging free. Padding is placed along each side of the pig to limit its attempts to roll laterally. Added supports are placed over the shoulders and behind the head to control movement of the anterior portion of the thorax and the head. During early T-2 administration trials some pigs would struggle and jerk their heads backward, causing the administration tubing to separate. The use of padded supports and securely clamping all tubing junctions effectively dealt with those problems.

Initially, tracheostomies were performed on the animals in order to provide a route of administration. This technique resulted in significant inflammatory responses at the surgical site and failure of cannula retention. It was determined that the stress produced would adversely affect experimental findings. A less invasive, more secure and effective method of aerosol

administration was adopted; oral intubation with endotracheal tubes having inflatable cuffs.

During the early trials, it was noticed that the endotracheal tube tended to accumulate mucoid secretions. It was difficult to assess the effect these secretions were having on the pigs during T-2 administration because the animals were enclosed in a chamber. In order to investigate this potential problem we anesthetized a pig, intubated it and allowed it to recover. Monitoring heart rate, respiratory rate and respiratory sounds over the trachea indicated that there was a progressive build-up of secretions in and around the tip of the endotracheal tube. When this occurred the pigs had difficulty breathing, struggled and had elevated heart rates. Attempts to control the secretions by intracheal administration of nebulized atropine were unsuccessful. It was found that suctioning of the endotracheal tube to remove the accumulated material alleviated the breathing difficulty and, thereby, stopped the struggling of the pig.

It is not possible to uncouple the tubing and insert a suction device while the pigs are in the enclosed chamber during T-2 administraion. To deal with this difficulty we modified our endotracheal tubes by inserting a much smaller diameter fenestrated tube through the wall of the endotracheal tube and added a suction line to the chamber. We can now intermittently suction the distal end of the tube while the T-2 toxin is being delivered to the pig. Since using this system we have not had problems with either accumulations of secretions or struggling by the pig. By eliminating the blockage we have also controlled one of the factors limiting toxin delivery to the lungs.

Characterization of the aerosol with respect to the T-2 concentration in the breathing air, the range of particle sizes and the mean aerodynamic diameter of the particles is an important part of determining both the dose and sites of respiratory tract deposition of the toxin (Phalen, 1984). In our

system, these parameters are determined by using data obtained from the cascade impactor and the mass concentration filter apparatus. The aerosol is sampled at constant times during the exposure period with each apparatus. The duration of the sampling interval must be varied with the concentration of T-2 toxin in the aerosol. Sampling times must be long enough to allow a measurable accumulation of material on the stages of the impactor, but short enough so that the pathways in the impactor are not blocked by the accumulated build-up of particles. The mass distribution of the particles deposited at the different levels of the impactor is gravimetrically determined by the use of a microbalance. The mass of T-2 on the individual coverslips at the different levels is used to define the particle size range and calculate a mass median aerodynamic diameter. The aerosol is also sampled with the mass concentration apparatus and the amount of toxin on its filter is used to calculate the concentration of T-2 per unit volume of air breathed by the pig.

An electrostatic point-to-plane precipitator has been acquired and is in the process of installation. It will be used to collect small amounts of the particles, so that they can be observed and measured using scanning and transmission electron microscopy. Observation of the generated particles will act as a check on the purity of the material that flows through the generating tubes to the animal. It will also serve as a check on the particle size distribution of the aerosol and a means of determining their shape.

Some of the problems encountered with the aerosol characterization system have included the following:

1) breakage of the substrates used to collect the particles at each stage of the cascade impactor;

2) inadequate sensitivity of the microbalance used to determine the mass of toxin on the substrates;

3) an electrical charge build-up on the substrates which interfered with the operation of the electrobalance;

4) sampling of wet particles.

We have developed methods of solving or controlling each of these difficulties.

As mentioned in last year's progress report, attempts to generate an aerosol using crystalline T-2 toxin as a starting material were unsuccessful. The effective method which we are now using, is nebulization of solutions of T-2 toxin dissolved in ethanol. An initial problem with this technique was the production of wet particles. Dry particles are desired because they are easier to size and they are likely to exert a greater effect on the respiratory tissues than particles in ethanol. A wet ethanol T-2 toxin aerosol would tend to be comprised of larger particles and would be less likely to penetrate to the alveolar level. In addition, the ethanol probably increases the tissue solubility of the T-2 toxin causing it to be more readily absorbed into the bloodstream. Efforts to control the wetting effect of the ethanol included use of an in-line dryer, zero-grade air and an in-line heater. The most effective technique involved the use of dried air and a higher ratio of dilution air flow to nebulization air flow. The addition of more dry air to the system caused the ethanol to evaporate, producing drier particles.

During development of the system a number of trials involving T-2 toxin administration to pigs were completed. One of the early exposures resulted in vomiting by the pig and changes in vascular perfusion, but a very large amount of toxin had to be nebulized to achieve this result. Attempts to produce clinical signs of T-2 toxicosis in the next six trials were uneventful. Some of the reasons for this have been mentioned previously in this section and include separation of the tubing and subsequent discontinuation of the trial,

of secretions in the endotracheal tube, a "wet" aerosol, and the generation of large particles which would not effectively penetrate to the aleveolar level of the lungs.

Deposition of particles in the generation and administration tubing was another obstacle. These portions of the system have been washed after each run in order to recover and quantitate the residual T-2 toxin. Results from these analyses demonstrated that much of the material generated was not reaching the pig. Improvements to increase the efficiency of the system have included the following:

1) Redirecting the tubing to shorten the path from the nebulizer to the pig;

2) The previously mentioned modification of the endotracheal tube to allow intermittent suctioning;

3) Utilizing a lower nebulization air flow;

4) Reduction of the T-2 concentration in the nebulizer.

The last two factors allow a longer exposure time and eliminate losses of T-2 toxin due to its being be pushed past the pig during the non-inspiratory phases of respiration. Table IC1 describes how the equipment and technique modificatons have reduced deposition of the toxin within the administration system.

Table IC1

Amounts of T-2 Toxin (mg) Recovered from the Inhalation System Before
and After System Modifications Described in the Report. Results Are
from Two Trials During each Period.

|  | Before(mean ± SD) | After(mean ± SD) |
|---|---|---|
| Nebulizer and Administration Tubing Rinse | 258.1 + 98.0 | 132.5 + 3.5 |
| Endotracheal Tube Rinse | 26.8 ± 12.5 | 2.5 ± 3.5 |
| Exhalation Tubing Rinse | 20.5 ± 12.2 | 8.0 ± 6.4 |
| Scrubber Filters | 15.2 ± 11.0 | 17.5 ± 24.7 |

Another modification of the equipment has involved the addition of an auxillary dry-air hose to the air reservoir bag of the generation system. This provides an extra source of air for the pig if its ventilation needs should exceed the baseline air flow delivery rate from the aerosol generator. This sytem will allow maintenance of fixed flow rates of both dilution air and nebulization air in the generation system. Previously, increases in the pig's ventilatory requirements necessitated periodic increases in dilutional air flow. This technique met the ventilation demands of the pig, but resulted ir air flow ratio changes during the nebulization characterization period. The new method allows rapid adaptation to the subject's needs, rapid equilibration of the system and conservation of the aerosol.

The equipment and techniques needed to administer T-2 toxin to swine by means of inhalation have been developed and refined. Procedural protocols have been written for the following:

1) animal preparations and handling;

2) equipment set-up;

3) toxin handling and safety measures;

4) equipment operation;

5) aerosol characterization;

6) clean-up of equipment and facilities after dosing.

METHODS

Eighteen hours before dosing, swine that are to be exposed to T-2 toxin by inhalation are weighed and allowed access to water but no food. On the morning of the treatment, the animals are anesthetized with halothane via a face mask and intubated. The endotracheal tube is secured and the pig is restrained in the sling suspension apparatus. The subject is allowed to recover from anesthesia and is placed in the inhalation chamber. A two-way, non-rebreathing valve is secured to the proximal end of the endotracheal tube

and the inhalation and exhalation hoses as well as the suction tubing are attached. Air is supplied to the pig to support respiration.

The individuals working in the room during the treatment period don the necessary protective clothing. Disposable plastic-coated outer wear covers the entire body except for a face mask. The masks have transparent shields and double breathing filters for protection against particles and chemicals.

All baseline physiologic measurements are determined. Then the T-2 solution is placed in the nebulizer and the aerosolization process is begun. One person monitors the pig and occasionally operates the endotracheal suction device. Another adjusts the controls of the equipment and collects the samples needed for characterization of the aerosol. A third fills in the experiment data sheet. The fourth person, who is in charge of the overall experiment, monitors all aspects. Another individual observes the procedure through a window from another room in case of a problem involving the toxic material.

The duration of the exposure period may be varied by controlling the T-2 concentration in, and the quantity of, the solution. After completion of nebulization, the pig is allowed to breath compressed air for ten minutes, thus flushing the system. The pig is then disconnected from the system, extubated and monitored.

After dosing, all sections of the generation and exhalation system are separately and thoroughly rinsed with ethanol. These rinses are saved for later T-2 analysis. When the radioactive isotope is used, the level of activity in each of the sections of equipment will be determined and the tubing will be rinsed, thereafter. Following the ethanol rinse, all of the tubing is washed with a solution of detergent, rinsed with water and allowed to dry. When the animal is removed from the room, the restraint equipment, chamber and floor are all treated with a clorox solution to inactivate any T-2

toxin and for disinfectiaon, thoroughly rinsed and allowed to dry. During the cleanup procedure the cascade impactor and mass concentration filter are disassembled and the substrates collected for later weighing and T-2 analysis. These data are then used to characterize the aerosol. Animals from the recent trials have been kept under observation until the clinical signs of T-2 toxicosis have subsided. The treated pigs that would be used for blood flow, physiology, pathology, and immunology studies would be appropriately monitored and handled as indicated in other portions of this report.

## RESULTS AND DISCUSSION

The goal in system design has been the production of clinical signs of T-2 toxicosis and subsequent death in a subject with an accurately determined dose of toxin. This goal has not been easily attained. As difficulties in aerosol characterization and pig restraint were overcome, other problems became apparent. Numerous trials have been conducted in order to develop appropriate methods of administration and characterization of T-2 aerosols. The system has been repeatedly adapted and improved to maximize exposure to the toxin and to increase the efficiency of dosing.

The in vivo nebulization trials and subsequent analytical results suggested that much of the aerosolized T-2 toxin was not reaching the pigs. Attempts were made to improve this situation by shortening the length, and altering the pathway of the tubing in the generation system. As these modifications were being made we had to establish that the system would:

1) generate a usable aerosol;

2) produce particles tnat were small enough to reach the alveoli in the lungs;

3) provide sufficiently efficient delivery of toxin to produce toxicosis.

In trial runs without a pig, the modified generation system satisfied the above requirements. At this time, one additional procedural change was instituted. Instead of shortening the dosing period, we attempted to lengthen it by doing the following:

1) using a less concentrated T-2 solution;

2) lowering the nebulization rate;

3) increasing the dilution air to nebulizer air ratio, but, nevertheless, lowering the dilution air flow rate to the minimum sufficient to supply the ventilatory demands of the pigs. These steps will limit the amount of T-2 toxin that bypasses the pig during the intervals when it was not in an inspiratory phase of respiration.

Recent inhalation exposures of experimental subjects to T-2 toxin have produced adverse clinical effects. Although we nebulized eight miligrams of T-2 toxin per kilogram of pig, our preliminary results indicate that less than 20% of that amount was retained by the animal. This was a sub-lethal dose, but it was followed by clinically apparent cyanosis within thirty minutes. After removal from the sling and placement in its pen, the first pig vomited twice, was anorexic, depressed and lethargic. Cyanosis and hypothermia were evident in the extremities, revealing clinically detectable hemodynamic abnormalities. Within six hours, severe diarrhea was evident and persisted until the next morning. Twenty-four hours after exposure, recovery was complete, based strictly on an assessment of clinical appearance.

We have demonstrated clinically apparent signs of T-2 toxicosis in swine by the inhalation route of exposure. We are still experiencing some retention of T-2 toxin in the generation system due to its adherence to the inflow tubing, along with the desired recovery in the scrubber system of that portion not impacting in the pig. Our results indicate that less than 20% of the eight milligrams of T-2 toxin per kilogram of pig that was nebulized, was

reaching the pig and caused signs of toxicosis. We believe it will be possible to improve upon toxin delivery by using a recently ordered nebulizer that will generate an aerosol at a slower flow rate than the current nebulizer. When that is done, the higher ratio of dilution air to nebulizer air will be used to thoroughly and rapidly evaporate the ethanol used to solubilize the T-2 toxin. That step, coupled with a decrease in the concentration of the T-2 in solution, should enable us to further decrease the size of T-2 toxin particles and thereby decrease adherence to the tubing of the generation system. This would further improve the efficiency of the system enabling more of the T-2 to reach the respiratory tract and penetrate more deeply into the lungs. Incorporation of the new nebulizer into the system and its testing should take two weeks.

Another important goal of this project is to establish a technique allowing accurate determination of the dose of T-2 toxin that is retained by the pigs. Currently we are able to recover and analytically quantitate the T-2 toxin retained that is left in the nebulization and collection systems. Difficulties with efficient recovery of the T-2 from the system limit the accuracy of this technique.

We believe it will be possible to validate a method to reduce the error in dosage calculation to a range of five to ten percent. Microcurie amounts of the high-energy, short half-lived, isotope of technetium (Tc 99m, 6-hr half-life) will be added to the T-2 solution used for aerosol generation. The dose administered to the pig will then be calculated by subtracting the amount of the isotope remaining in the generation and scrubber systems from the initial quantity nebulized. This technique has frequently been used in calculating doses of substances administered by inhalation (Brain and Valberg, 1979). It is more accurate and reproducible than other methods because recovery efficiencies are less of a problem. Results from similar systems

suggest that particles from two simultaneously nebulized materials maintain their relative ratios. There are, however, several steps that must be taken to validate the system. These would include the following:

1) establishing that the isotope and T-2 would be distributed by the system and utilized by the pig in a similar fashion. This would be done by measuring and comparing the ratio of radioactivity to T-2 toxin in the nebulizer, cascade impactor, generated aerosol and exhaled air of the pig;

2) measurement and correction for gamma ray attenuation effects caused by the different materials used in the tubing system;

3) standardization of the counting efficiency.

If there are no major problems with the system, validation should be completed by mid-October, 1984.

Although we could estimate dose delivery strictly by the use of the cascade impactor, mass concentration filter, and by analyzing the wash solutions from the system, recovery efficiency of the washes is likely to be variable enough to warrant the effort placed in validating a more accurate technique. More accurate determination of the dose retained by the pig's respiratory tract will allow better comparisons with other dosage routes and more meaningful dose-response comparisons.

SUMMARY

Considerable effort has been invested in developing a system for studying the effects of inhaling an aerosol of T-2 toxin on swine. Techniques have been worked out and protocols developed for the following:

1) animal restraint and monitoring;

2) toxin handling and administration;

3) aerosol generation and clean-up;

4) aerosol characterization.

These efforts have resulted in the ability to produce clinically apparent signs of T-2 toxicosis by the inhalation route of exposure. There are continuing efforts to increase the efficiency of toxin delivery. Although we are able to estimate the dose of toxin retained by the pigs, a more reproducible and accurate method, using a radioactive isotope, is under development. This technique will allow better comparisons with the other dosage routes and more meaningful dose-response relationships.

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D. TESTING OF POTENTIAL THERAPEUTIC AGENTS FOR T-2 TOXICOSIS--Robert H. Poppenga

OBJECTIVES

1. To determine the role of alpha adrenergic stimulation during T-2 toxicosis by evaluating the efficacy of alpha adrenergic blockade in controlling circulatory shock in this syndrome.

2. To determine whether a thromboxane synthetase inhibitor is successful in preventing production of thromboxane $A_2$ and in alleviating circulatory shock and death attributable to T-2 toxicosis.

3. To evaluate the effectiveness of pharmacologic doses of a glucocorticoid in the shock syndrome occurring during T-2 toxicosis.

4. To identify a sound therapeutic regimen to combat the circulatory shock induced by T-2 toxin.

5. To evaluate other pharmacologic compounds hypothesized to be of benefit in reversing or ameliorating the adverse effects following acute exposure to T-2 toxin.

HYPOTHESES TO BE TESTED

1. Administration of an inhibitor of thromboxane $A_2$ ($TXA_2$) production will prevent increases in thromboxane $B_2$ ($TXB_2$) (metabolite of $TxA_2$) in T-2 toxicosis and this prevention will be correlated with decreased adverse circulatory effects during T-2 toxicosis.

2. Alpha adrenergic blockade in animals affected by acute T-2 toxicosis improves circulation by alleviating splanchnic and peripheral vasoconstriction and may decrease myocardial damage.

3. High doses of corticosteroids are effective in reducing the shock syndrome of T-2 toxicosis and this is correlated with reduced appearance of thromboxane $B_2$.

4. Thromboxane synthetase inhibition, alpha-adrenergic blockade and corticosteroids are all effective in lessening myocardial decompensation in the later stages of T-2 toxicosis.

5. A calcium channel blocker, a narcotic antagonist, ascorbic acid, adenosine triphosphate (ATP) or ATP with magnesium chloride, a protease inhibitor, dimethylsulfoxide, n-acetyl cysteine, and/or trichodermin, will alleviate T-2 toxicosis.

INTRODUCTION

Based upon our current knowledge of the pathophysiology of acute T-2 toxicosis in swine (Lorenzana, et al. Appendix A and B), the drugs thus far selected for evaluation can be arbitrarily placed into one of three categories:

A. Those agents which may be useful in a general supportive role.

B. Those agents which have known, specific sites of action and whose selection was based on their anticipated ability to reverse the adverse effects of T-2 toxin upon animal physiology or based on their anticipated ability to influence processes involved in T-2 metabolism.

C. Those agents with either specific or general actions which might play a beneficial role based upon hypothesized T-2 toxin pathophysiologic mechanisms.

Since acutely dosed swine experience a lethal shock syndrome (Beasley et al., Lorenzana et al., (Appendix A, B, and C), our initial efforts have focused on evaluating those compounds which have been proven or are speculated to be of benefit in treating shock due to a variety of etiologies. However, other potentially useful agents with properties not related to shock therapy have also been considered.

For the majority of compounds selected for evaluation, a thorough literature review was conducted in order to gain an understanding of their previous therapeutic uses and pharmacokinetics. This, in turn, allowed formulation of reasonable dosage regimens.

The agents thus far evaluated on a preliminary basis in either rats or swine are listed below. They are grouped according to the above mentioned categorization scheme.

A. General--Supportive

    1. Methylprednisolone(MP)--a glucocorticosteroid, SoluMedrol[R]--Upjohn

        a. Methylprednisolone has been proven to be of benefit in a variety of shock states (Lefer et al., 1977).

        b. Its primary beneficial effect in shock, although uncertain, is probably related to the maintenance of cell integrity (Spath et al.).

    2. Ascorbic acid--Vitamin C--Scorbate[R]--Burns-Biotec

        a. Vitamin C is theorized to be of benefit due to its general antioxidant action.

    3. Adenosine triphosphate (ATP) (crystalline disodium salt of ATP, 99-100% pure and derived from equine muscle), alone or in combination with magnesium chloride ($MgCl_2$)--Sigma.

        a. $ATP-MgCl_2$ has been shown to enhance survival in both hemorrhagic and endotoxic shock models (Chaudry, Kraven, Filkins et al.).

        b. Believed to be of benefit by providing ischemic cells with a readily utilizable energy source (Chaudry).

B.  Specific--Based Upon Known Pathophysiology and Metabolism

　　1.  Dazemgrel (UK-38,485)--a thromboxane synthetase inhibitor--Pfizer

　　　　a.  Refer to Intravascular Studies (IA) for the apparent role of thromboxane in acute T-2 toxicosis.

　　　　b.  This agent would serve to counteract thromboxane $A_2$'s potent vasonstrictor and platelet aggregating ability (Needleman et al.).

　　　　c.  May enhance prostacyclin synthesis which would thereby decrease coronary vascular resistance, stabilize lysosomal membranes and inhibit both proteolysis and catecholamine synthesis (Cowley et al.).

　　2.  Phenoxybenzamine--a nonspecific alpha-adrenergic blocker--Dibenzyline[R]--Smith, Kline, French.

　　　　a.  Phenoxybenzamine could potentially counteract coronary and peripheral vasoconstriction caused by catecholamine release and thereby alleviate myocardial and visceral organ ischemia.

　　3.  Trichodermin--a trichothecene mycotoxin

　　　　a.  Trichodermin is a much less acutely toxic trichothecene mycotoxin as compared to the structurally related T-2 toxin (rat subQ $LD_{50}$ for trichodermin is 500-1000 mg/kg).

　　　　b.  Hypothesized to act as a competitive inhibitor of T-2 toxin binding to peptidyl transferase on ribosomes, a process which results in protein synthesis inhibition. Also hypothesized to preserve polyribosome profiles, thereby preventing m-RNA degeneration by endogenous cellular RNA-ases during T-2 toxin metabolism and excretion.

C.  Specific or General--Based Upon Hypothesized Pathophysiologic Mechanisms

　　1.  Naloxone--an opioid antagonist Narcan[R]--Endo Pharmaceuticals.

a. There are reports of beneficial results following naloxone administration in hemorrhagic and endotoxic shock (Reynolds et al. and Curtis et al.).

b. It would antagonize endogenously released opioids, provide a positive inotropic effect, and stabilize lysosomal membranes (Holaday and Curtis et al.).

2. Diltiazem--a slow calcium channel blocker--Cardizem[R]--Marion.

a. Diltiazem may prevent the intracellular calcium overload in cardiac and smooth muscle which occurs following prolonged ischemia thereby prolonging cell viability.

b. May result in beneficial hemodynamic effects such as vasodilatation, slowing of the heart rate, and maintenance of cardiac contractility (Hacker et al.).

3. Aprotinin--a protease inhibitor--Sigma Chemical (also available as Trasylol[R] from Mobay Chemical).

a. The primary benefit of aprotinin would be to inhibit zymogenic and lysosomal proteases which are catalysts in the formation of toxic shock factors such as myocardial depressant factor (MDF) (Lefer, 1972 et al.).

4. Dimethylsulfoxide 90%--DMSO--Burlington Biomedical Corporation

a. Dimethylsulfoxide is proposed to be useful for tissue protection against ischemia.

b. May stabilize lysosomal membranes and has free radical scavenging ability (Kharasch et al.).

5. N-acetylcysteine--Mucomyst[R]--Mead-Johnson

a. Glutathione conjugation is a major detoxification pathway in the body. Hepatic concentrations of glutathione have been shown to decline following the administration of T-2 toxin. As in the treatment of other

toxicoses, N-acetylcysteine could serve as a source of cysteine, which is the limiting amino acid in the synthesis of new glutathione.

The above list is by no means comprehensive. As will become evident, many of the preceding agents, when used alone, had no effect on survival time or survival rate in rats following iv administration of T-2 toxin. Therefore, other approaches such as the use of sodium bicarbonate to counteract the severe lactic acidosis which occurs in acutely dosed swine (Lorenzana et al., Appendix A) or the oral administration of activated charcoal to modify possible enterohepatic circulation of T-2 excreted in the bile will be emphasized in work to be completed in the next 6 months. Also the use of balanced electrolyte solutions, which have proven to be a mainstay of most shock treatment protocols, will receive considerable attention.

## METHODS

Originally it was believed that a dual approach to drug evaluation would be most effective. This would involve the initial screening of a greater number of agents using rats as the experimental animal, with those agents showing efficacy being formally studied utilizing swine. Use of the rat would be more cost effective and would allow for the use of larger numbers of animals with percentage survival and survival time as the variables of interest. Subsequent use of swine would then permit in-depth studies employing physiologic monitoring and multiple sample collections. It was concluded, however, that certain potential therapeutic agents should be initially evaluated in swine, rather than rats, due to the necessity of adjusting dosage over time, based upon repeated measurements of specific physiologic parameters. The alpha-adrenergic blocker, phenoxybenzamine, which causes peripheral vasodilation, serves as an example. In this case arterial blood pressure must be monitored during phenoxybenzamine administration and,

if required, the rate of administration rate altered and/or parenteral fluids given in order to maintain adequate arterial blood pressure.

A. Rat Studies

All of the above-listed compounds, with the exception of phenoxybenzamine, were evaluated in groups of rats. Female, Sprague-Dawley rats approximately 225 to 240 grams were used. The protocol called for the administration of T-2 toxin at 1 mg/kg via the tail vein. With the exception of a predosed group of animals in the study of trichodermin, all potential therapeutic compounds were administered intraperitoneally immediately following the intravenous administration of T-2 toxin. As mentioned earlier, dosage regimens, where possible, were based upon the pharmacokinetics or the previous uses of particular compounds as reported in the scientific literature. For some agents empirical dosages were utilized.

When agents requiring multiple dosings were used, treatment was continued for a minimum of 5 hours. Based upon published pharmacokinetic data, dosages were chosen to maintain therapeutic blood levels for a variable amount of time beyond the 5 hour treatment period.

The exact dosage regimens are given in Tables IB1-5 presented below, under Results/Discussion, along with the mean survival time for the various groups. Where multiple dosings required multiple animal handlings, an additional positive (T-2 dosed) control group was included. This group was given ip saline injections as often as the most frequently handled treatment group. This was done to assess any adverse stress effects from the additional handling.

All animals were observed until death or 48 hours post-dosing at which time survivors were killed. A wide variety of tissues were saved in 10% buffered formalin for possible future histopathologic evaluaton.

B.  Swine Study

As mentioned previously, swine were used for the initial assessment of the efficacy of phenoxybenzamine. Female, 50 kg crossbred swine were fully instrumented as described elsewhere (Lorenzana et al., Appendix A and B), lactic acid, blood gases, dye dilution, cardiac output, and the mean and phasic pressures in the aorta, pulmonary artery , and left atrium. and various hemodynamic parameters were measured.

Two treatment animals received T-2 toxin iv at 2.4 mg/kg B.W. (2 x $LD_{50}$ dose) followed immediately by 1 mg/kg B.W. phenoxybenzamine in 200 ml Ringer's solution which was infused over a 1 to 2 hour period. A third animal served as a positive control; being given T-2 toxin but no phenoxybenzamine.

Following the administration of phenoxybenzamine, a slow intravenous drip of Ringer's solution was begun and was maintained throughout the initial 8 hour observation period. Lactic acid, blood gas, and hemodynamic parameters were measued at 1/2 hour intervals for the first 4 hours, hourly thereafter until 8 hours post-dosing and then again 24 hours post-dosing. All 3 swine were observed for a 24-hour period at which time they were humanely killed for necropsy. Representative tissues were saved for histopathology.

Since so few animals were used, definitive conclusions cannot be drawn and therefore individual data will not be included. However, some notable clinical impressions are reported in the Results/Discussion Section below.

RESULTS AND DISCUSSION

Tables ID1-5 present the rat study results.

Based upon the data obtained in rats, only methylprednisolone (MP) was proven to be of significant benefit following acute iv administration of T-2 toxin. Its exact mechanism of action is unclear but is probably related to the preservation of cell membrane integrity.

The other significant finding was an apparent additive or synergistic effect of the trichothecene trichodermin with T-2 toxin when trichodermin was administered ip either one hour prior to, or immediately following an iv dose of T-2 toxin. Although both trichodermin and T-2 toxin inhibit peptidyl transferase, which is a crucial enzyme for proper protein synthesis, trichodermin is primarily an inhibitor of termination as opposed to T-2's inhibition of initiation. A combined effect may explain the adverse of trichodermin on the T-2 toxin dosed animals. These results might also predict the failure of other trichothecene inhibitors of either elongation (such as verrucarol) or termination processes of protein synthesis to prevent or alleviate T-2 toxicosis.

Table ID1

Effects of Saline, Naloxone, Diltiazem, and Methylprednisolone
on Survival Time of T-2 Toxicosis in Rats

| Group | Dosage Regimen | Mean Survival Time (HR) ± S.E.M. |
|---|---|---|
| T-2 Control | 1 mg/kg IV | 14.11 ± 0.96 N = 8 |
| T-2 + Saline | .25 cc saline IP each hour for 5 hours | 25.63 ± 6.57 N = 8 |
| T-2 + Naloxone | 10 mg/kg IP each hour for 5 hours | 13.68 ± 1.48 N = 8 |
| T-2 + Diltiazem | 18 mg/kg IP every 2 hours for 3 treatments | 13.56 ± 0.56 N = 8 |
| T-2 + MP | 30 mg/kg given once | 41.57 ± 4.24 N = 8 |

Table ID2

Effects of Saline, Dazemgrel, N-Acetyl-Cysteine and DMSO
on Survival Time of T-2 Toxicosis in Rats

| Group | Dosage Regimen | Mean Survival Time (HR) ± S.E.M. |
|-------|----------------|----------------------------------|
| T-2 Control | 1 mg/kg IV | 12.91 ± 1.13 N = 6 |
| T-2 + Saline | .25 cc saline IP each hour for 5 hours | 12.79 ± 1.43 N = 7 |
| T-2 + Dazemgrel | 25 mg/kg IP initial dose followed by 10 mg/kg every hour for 5 hours | 11.95 ± 0.33 N = 10 |
| T-2 + N-Acetyl-Cysteine | 140 mg/kg IP initial dose followed by 70 mg/kg 5 hours later | 11.37 ± 0.17 N = 6 |
| T-2 + DMSO 30% | 1 gram/kg IP given once | 14.49 ± 2.62 N = 7 |

Table ID3

Effect of Trichoderm on Survival Time of T-2 Toxicosis in Rats

| Group | Dosage Regimen | Mean Survival Time (HR) $\pm$ S.E.M. |
|-------|----------------|--------------------------------------|
| T-2 Control | 1 mg/kg IV | 22.47 $\pm$ 4.84 N = 7 |
| Trichodermin Pre-Treat. | 100 mg/kg IP/1 hour prior to dosing w/T-2 | 11.26 $\pm$ 1.15 N = 8 |
| Trichodermin Post-Treat. | 100 mg/kg IP immediately following dosing w/T-2 | 10.41 $\pm$ .63 N = 8 |

Table ID4

Effects of ATP and ATP Plus Magnesium Chloride on Survival Time
of T-2 Toxicosis in Rats

| Group | Dosage Regimen | Mean Survival Time (HR) ± S.E.M. |
|---|---|---|
| T-2 Control | 1 mg/kg IV | 14.85 ± 2.70 N = 8 |
| ATP | Immediately following T-2: .25 ml ATP solution (200 uMol/ml) divided into equal parts and administered IP 15 min. apart | 12.20 ± .37 N = 8 |
| ATP-MgCl$_2$ | Immediately following T-2: .25 ml ATP & MgCl$_2$ solution (100 uMol ATP +) divided into equal parts & administered IP 15 min. apart | 14.06 ± 1.57 N = 7 |

Table ID5

Effects of Aprotinin and Vitamin C on Survival Time
of T-2 Toxicosis in Rats

| Group | Dosage Regimen | Mean Survival Time (HR) ± S.E.M. |
| --- | --- | --- |
| T-2 Control | 1 mg/kg IV this control group was given .25 ml saline 2 hours post-dosing | 11.76 ± .62 N = 7 |
| Aprotinin | 15,000 KIU/Kg ip initial dose following T-2 then 10,000 KIU/Kg ip at 2 hours post-T-2 dosing | 14.01 ± 1.62 N = 8 |
| Vitamin C | 500 mg/kg ip immediately after T-2 | 13.32 ± 1.02 N = 8 |

In the two swine treated with phenoxybenzamine, there was a significant delay in the onset and severity of diarrhea compared to the control. Skin color and capillary refill time, both indicative of peripheral perfusion, were greatly improved in the two treatment animals vs the control. This indicates a lessening in the degree of peripheral vasoconstriction previously observed during pathophysiology studies. Urinary output was maintained to a greater degree in the treatment animals. Alertness was also improved throughout the observation period. In summary, although all three animals showed relatively severe physiologic changes characteristic of T-2 toxicosis (decrease in cardiac output, mean arterial blood pressure and a severe lactic acidosis), in certain respects, the two treatment swine were less clinically affected than the positive control animal.

Based on a degree of clinical improvement with phenoxybenzamine in T-2 dosed swine, alpha-adrenergic blockers should be investigated further. High plasma concentrations of catecholamines have been measured in swine following acute, iv administration of T-2 toxin (Lorenzana et al., Appendix _). The catecholamine interaction with alpha-adrenergic receptors in vascular smooth muscle may account, in part, for the severe peripheral vasoconstriction which occurs in T-2 toxicosis. The blockade of this interaction may be responsible for the beneficial effect of phenoxybenzamine.

It has also been speculated that elevated catecholamine concentrations adversely affect the heart (Lehr) and may account for some of the striking heart lesions reported by Dr. Pang in our pathology study (Sections II and III). It would seem logical in further studies therefore, to combine an alpha$_1$-blocker, such as prazosin with a beta-blocker, such as propanolol to assess their ability to protect the cardiovascular system from the acute effects of T-2 toxicosis.

One last point; because a particular agent did not prove to be of benefit by itself, it does not necessarily follow that it could not be beneficial in combination with other drugs. For example, although dazemgrel (a thromboxane synthetase inhibitor) did not prolong or enhance survival in treated rats, it may be useful in swine. This may occur if, glucocorticosteroid does not adequately prevent the elaboration of thromboxane $A_2$ following T-2 administration. The combination of a glucocorticosteroid and a thromboxane synthetase inhibitor may then prove to be of more benefit than either agent used alone.

## FUTURE WORK

It is anticipated that a formal therapeutic study utilizing swine will begin within 60 days and be completed by March 31, 1985. The following treatment groups will be evaluated:

A. Group I - T-2 positive control,

B. Group II - T-2 and a baseline treatment consisting of methylprednisolone. sodium bicarbonate, and a balanced electrolyte solution.

Rationale:

1) This would constitute classical shock therapy and should be the standard against which other therapeutic protocols are compared.

2) Glucocorticosteroids have been shown to enhance survival in rats following acute iv administration of T-2 toxin.

3) We know T-2 exposed swine became quite acidotic (Lorenzana et al., Appendix A and B).

4) As with other shock states, T-2 induced shock should cause a decrease in venous return of blood to the heart, therefore, enhancement of venous return by the administration of a balanced electrolyte solution is warranted.

5) If these agents used in combination show promise there is justification to use them in the interim should trichothecenes be used as chemical warfare agents in the immediate future pending assessment of other preventive or treatment approaches.

C.  Group III - T-2 and an alpha-adrenergic blocker such as prazosin in combination with a balanced electrolyte solution

Rationale:

1)  See Results/Discussion.

2)  A balanced electrolyte solution must be administered along with the alpha-adrenergic blocker to compensate for possible lowering arterial blood pressure subsequent to vasodilatation.

D.  Group IV - T-2 and a beta-adrenergic blocker such as propanolol.

Rationale:

1)  A beta-adrenergic blocker would serve to protect the myocardium from excessive levels of catecholamines which are released in swine following iv administration of T-2 toxin.

2)  There is histopathologic evidence of myocardial lesions compatible with catecholamine-induced necrosis as reported in Sections II and III.

E.  Group V - T-2 and clonidine.

Rationale:

1)  Clonidine may be beneficial by decreasing peripheral sympathetic tone via centrally mediated mechanisms [Brave].

A dosage of 3.6 mg/kg T-2 given iv will be used for all swine.

A number of parameters will be measured including, but not restricted to:

1)  Blood gases.

2)  Hemodynamic parameters such as cardiac output and mean arterial blood pressure in addition to central venous pressure.

3)  Serum chemistries, including amylase and lipase.

4) Complete blood count.

5) Electrocardiogram.

6) Catecholamines, thromboxane and prostacyclin

7) Lactic acid

8) Cardiac bulk electrolytes

In addition, a complete necropsy will be performed with a number of of tissues saved for histopathology. Control and treatment animals will be observed and evaluated for up to 48 hours in order to assess any post-treatment complications.

During the study period any new treatment possibilities will be preliminarily evaluated in rats as before. Promising agents will be incorporated into treatment protocols in swine at a future date.

VIII. SUMMARY

All of the drug agents previously proposed for the treatment of T-2 toxicosis have been screened for efficacy in either rats or swine. A number of other agents have also been assessed. Only the glucocorticosteroid, methylprednisolone, and the alpha-adrenergic blocker, phenoxybenzamine, utilized in rats and swine respectively have shown any degree of efficacy. The original objective of assessing the effects of various drug agents on regional blood flow will be postponed until definitive treatment protocols are formulated.

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E. STUDIES ON THE IN VITRO AND IN VIVO ADSORPTIVE CAPACITY OF ACTIVATED CHARCOALS FOR T-2 TOXIN AND OTHER TOXICANTS--Paula M. Bratich (Research Funded by the National Animal Poison Control Center-- University of Illinois)

OBJECTIVES

1. To determine the in vitro adsorptive capacity of "superactive"[*] charcoal, Calgon Activated Carbon[**], and Toxiban[***] for T-2 toxin, carbaryl, chlorpyrifos, nitrite and strychnine.

2. To determine whether in vivo efficacy of the charcoals for the given toxicants can be predicted from in vitro adsorption studies.

3. To determine the in vivo efficacy of charcoals in rats dosed with the above listed toxicants.

4. To determine the optimum dosage of charcoal to prevent absorption of a given toxicant by the animal.

INTRODUCTION

In human and animal poison cases, emergency care routinely involves prevention of absorption, enhancement of drug elimination, life sustaining supportive care, and specific antidotal treatments, if available. Inhibition of drug absorption prevents toxicants and their metabolites from entering the circulation, and is, thus, the first line of treatment.

Levy (1982) indicates that activated charcoal not only inhibits a wide variety of drug atsorption from the gastrointestinal tract but also increases the clearance of drugs that have already been absorbed and are in the systemic circulation. However, the major advantage of administering activated charcoal

[*]Supersorb[R], Amoco Research Corporation, Chicago, IL 60601, (Manufactured by Anderson, Development Company, Adrian, Michigan and Distributed by Med-Corp. Inc., Dallas, TX)
[**]Calgon Activated Carbon, P. O. Box 1346, Pittsburg, PA 15230
[***]Toxiban[R] Vet-A-Mix, Inc., Shenandoah, IA

is that this antidote can be given immediately at home or in the field (if the patient is not unconscious), or at the physcian's or veterinarian's office.

The adsorptive capacity of different grades and brands of activated charcoal have been tested. Recently, experimental charcoals having very high surface areas have been developed by the Amoco Research Corporation and are being produced by Anderson Developmental Company.

These new "superactive" charcoals have three times the surface area of conventional activated charcoal (Cooney, 1980). One would thus expect the "superactive" charcoals to adsorb three times as much toxin. In vitro experiments by Cooney (1977) on aspirin adsorption from simulated gastric fluid by a proven conventional activated charcoal, Norit A, and an Amoco "superactive" charcoal confirm these expectations. Human studies by Chung, Murphy and Taylor (1982) showed that for an equivalent weight of "superactive" charcoal administered, 1.7 times as much aspirin was adsorbed by the "superactive" charcoal.

Preliminary work as described below at the University of Illinois National Animal Poison Control Center also provides evidence of the superior adsorptive capacity of this "superactive" charcoal when compared to a commonly used activated charcoal and a commercial charcoal suspension sold for veterinary use.

METHODS

1. In vitro analysis examining the adsorptive capacity of charcoal for T-2 toxin, carbaryl, chlorpyrifos, nitrite and strychnine.

Three levels of each toxin and three levels of each charcoal in addition to appropriate controls were assayed. The volumes of toxicants and adsorbents used in the in vitro studies were the same as used to dose a 250 g rat. (Rats were given a calculated dose of charcoal of approximately 1 g/kg bw.) The

calculated volumes were as follows: 2.5 ml of charcoal suspension, 1 ml of toxin and 1.5 ml of water.

Four dose levels of each toxicant were examined:

1)   the control;

2)   the dose(s) of toxicant calculated to be the same concentration(s) as administered in preliminary $LD_{100}$ studies. This would comprise the dose at which one would expect all of the positive control animals (animals given only the toxicant) to die.

3)   one-half the dose determined in number 2 above;

4)   three times the dose determined in number 2 above.

Four levels of the charcoals were used:

1)   none (control);

2)   the current recommended dose (1g/kg equivalent);

3)   one-half of the current recommended dose;

4)   three times the current recommended dose. (Toxiban[R] could only be used at 104 mg/ml concentration, because it is a pre-packaged liquid suspension.) The remainder of the _in vitro_ procedure varied with each toxicant.

a)   Nitrite

The charcoal, nitrite and water were added to tubes and then rocked mixed) for thirty minutes before centrifugation. A small amount of the supernatant was then removed, diluted and analyzed. The procedure for analysis of nitrite was a modification of Method 354.1 from _Methods for Chemical Analysis of Water and Wastes (EPA-600/4-79-020)_.

b)   Carbaryl

The formulation of carbaryl used was Ortho Liquid Sevin[R*]. The charcoal, carbaryl and water were placed in tubes and a rocker mixer was used for thirty minutes. Thereafter, a 0.2 ml aliquot of the mixture was removed, diluted

with 0.8 ml acetylnitrile, filtered and assayed by high pressure liquid chromatography (HPLC). The three levels of carbaryl analyzed were 25 mg/ml, 75 mg/ml, and 284 mg/ml. The samples were assayed on um 10 u Perkin Elmer C18 column with a Perkin Elmer Series 4 HPLC and 254 nm fixed wavelength UV detector. The solvent system was acetonitrile and water (1 + 1) and the flow rate was 2 ml/min.

c) Strychnine

The strychnine formulation used was Mallinckrodt Strychnine Sulfate Purified Powder. Charcoal, strychnine and water were added to tubes, mixed for thirty minutes and a 0.25 ml aliquot of the mixture was removed, diluted with 1.0 ml of methanol, filtered and analyzed by HPLC. The three concentrations of strychnine sulfate assayed were 2 mg/ml, 4 mg/ml and 10 mg/ml. After assessment of the above three concentrations, it was decided that a fourth, 25 mg/ml, should be tested. The HPLC apparatus was the same as described for carbaryl. A 60% MeOH and 40% potassium dihydrogen orthophosphate solvent system was used at a flow rate of 1.5 ml/min.

d) Chlorpyrifos

The formulation of chlorpyrifos used was Ortho Lawn[R*] Spray . The charcoal, chlorpyrifos and water were mixed for thirty minutes and a 0.25 ml aliquot of the mixture was removed, diluted with 1.0 ml isopropanol, filtered and assayed by HPLC. The three concentrations of chlorpyrifos analyzed were 40 mg/ml, 26 mg/ml and 14 mg/ml. The HPLC apparatus was the same as described for carbaryl. The solvent system was changed to methanol and water (4 : 1) with a flow rate of 2.0 ml/min.

e) T-2 Toxin

The T-2 in vitro work has not been completed. However, the procedure being used is similar to those listed above. The samples are being analyzed using a 10 u Perkin Elmer C18 column with a Perkin Elmer Series 4 HPLC. Due

to the lack of any significant UV absorbance by T-2, a refractive index detector was used. Optimization of the chromatographic conditions is in progress. Concentrations to be assayed will include 1 mg/ml, 2 mg/ml, and 8 mg/ml (equivalent to 1x, 2x, and 8x the concentrations used in $LD_{50}$ studies of T-2 toxin in 250 g rats in preliminary studies).

2. In vivo study examining the efficacy of charcoal for T-2, carbaryl and strychnine.

In preliminary work, a dose was found in the rat which was lethal to all positive controls (no adsorbents, toxicants only), but which allowed differentiation in the survival rate of rats given the charcoal treatments. When the lethal dose ($LD_{100}$) was found, 40 rats were divided into four groups of ten each for each toxicant studied. Three different charcoal preparations ("superactive", Calgon and Toxiban[R]) were used in addition to positive controls (toxicant only). The toxicants used were from the same container as those used in the in vitro studies. The survival rate was recorded daily for seven days after administration of the toxicant and treatment.

RESULTS

1. In Vitro

   a) Nitrite

   None of the three charcoals investigated displayed any significant capacity for adsorting nitrite.

   b) Carbaryl

   The three brands of charcoal and their adsorptive capacity for carbaryl are given on Table III. With the low concentration of carbaryl, the low doses of the different charcoals did not differ in their adsorptive capacities. At the high concentration of carbaryl and the low dose of charcoal, none of the charcoals were effective, with only 10% adsorption. However, at the high dose

of carbaryl and the high dose of charcoal, "superactive" charcoal adsorbed 150% of that adsorbed by Calgon activated charcoal.

c) Strychnine

The adsorption of the various concentrations of strychnine by the different concentrations of activated charcoal are given in Table IE2.

## Table IE1

### The Adsorptive Capacity of Different Charcoals for Carbaryl at Three Different Concentrations

| Concentration of Charcoal (mg/ml)[*] | Brand | Fraction of Carbaryl Adsorbed (Percent) Initial Concentration of Carbaryl[**] | | |
|---|---|---|---|---|
| | | 25 mg/ml | 75 mg/ml | 284 mg/ml |
| 52 | Calgon | 71 | 20 | 10 |
| | Amoco | 71 | 46 | 9 |
| 104 | Calgon | 70 | 46 | 8 |
| | Amoco | 86 | 72 | 20 |
| | Toxiban | 11 | None | 14 |
| 250 | Calgon | 94 | 75 | 40 |
| | Amoco | 100 | 94 | 61 |

[*] The volume of all 3 charcoal suspensions was 2.5 ml.

[**] The volume of all 3 carbaryl suspensions was 1 ml.

Table IE2

Adsorptive Capacity of Charcoals for Strychnine
at Different Concentrations

| Concentration of Charcoal (mg/ml)[*] | | Fraction of Strychnine Absorbed (Percent) | | | |
| | | Initial Concentration of Strychnine[**] | | | |
| | Brand | 2 mg/ml | 4 mg/ml | 10 mg/ml | 25 mg/ml |
|---|---|---|---|---|---|
| 52 | Calgon | 100 | 100 | 94 | 79 |
| | Amoco | 100 | 100 | 99 | 98 |
| 104 | Calgon | 100 | 100 | 99 | 90 |
| | Amoco | 100 | 100 | 99 | 98 |
| | Toxiban | 100 | 100 | 100 | 93 |
| 250 | Calgon | 100 | 100 | 100 | 99 |
| | Amoco | 100 | 100 | 100 | 99 |

* The volume of all 3 charcoal suspensions was 2.5 ml.
** The volume of all 3 strychnine suspensions was 1 ml.

All three brands of charcoal appeared effective in their adsorptive capacity for strychnine. However, at the highest concentration of strychnine and the lowest concentration of charcoal, "superactive" bound 125% of that adsorbed by Calgon activated charcoal.

d, Chlorpyrifos

The adsorption of the various concentrations of chlorpyrifos by the different concentrations of charcoal are given in Table IE3. A discrepancy was noted for the "superactive" charcoal at the 250 mg/ml concentration since a greater fraction of the insecticide was adsorbed at a chlorpyrifos concentration of 40 mg/ml, than at 26 mg/ml. The 250 mg/ml charcoal concentration will be repeated for chlorpyrifos. Except for the discrepancy mentioned above, "superactive" charcoal had 2-3 times the adsorptive capacity of the Calgon charcoal.

## Table IE3

### Adsorptive Capacity of Charcoals for Chlorpyrifos
### at Three Different Concentrations

| Concentration of Charcoal (mg/ml)* | | Fraction of Chlorpyrifos Absorbed (percent) | | |
|---|---|---|---|---|
| | | Initial Concentration of Chlorpyrifos** | | |
| | Brand | 14 mg/ml | 26 mg/ml | 40 mg/ml |
| 52 | Calgon | 100 | 19 | 16 |
| | Amoco | 100 | 67 | 48 |
| 104 | Calgon | 100 | 36 | 25 |
| | Amoco | 100 | 100 | 84 |
| | Toxiban | 50 | 53 | 64 |
| 250 | Calgon | 100 | 62 | 55 |
| | Amoco | 100 | 64 | 100 |

* The volume of all 3 charcoal suspensions was 2.5ml.
** The volume of all 3 chlorpyrifos suspensions was 1 ml.

2. _In vivo_ Studies testing the efficacy of various charcoals in the protection of rats given lethal doses of strychnine, carbaryl, and T-2 toxin.

Studies with rats have been done with strychnine, carbaryl and T-2 toxin. Strychnine (4 groups of 10 rats/group) was given orally at a dose of 65 mg/kg. Carbaryl (10 rats/group) was given orally at a dose of 300 mg/kg and T-2 toxin (6 rats/group) was given orally at 8 mg/kg. Rats dosed with strychnine or carbaryl were treated immediately with "superactive" charcoal, Calgon activated charcoal, Toxiban[R] or water. T-2 dosed rats were treated immediately with either "superactive" charcoal or water. The data is given in Table IE4.

"Superactive" charcoal treated rats exhibited an increased survival rate by at least 2-3x as compared to Calgon activated charcoal and Toxiban[R]. The positive controls all died in each study.

Table IE4

Efficacy of Activated Charcoals in the Protection
of Rats Given Lethal Doses of
Strychnine, Carbaryl, and T-2 Toxin

| | Survivors/Animals Dosed[*] | | | |
| | Activated Charcoal Treatment | | | |
| Toxicant (concentration) | Calgon (104 mg/ml) | Supersorb (104 mg/ml) | Toxiban (104 mg/ml) | Control[**] |
| --- | --- | --- | --- | --- |
| Strychnine (65 mg/kg) | 4/10 | 9/10 | 3/10 | 0/10 |
| Carbaryl (300 mg/kg) | 1/10 | 10/10 | 2/10 | 0/10 |
| T-2 (8 mg/kg) | – | 5/6 | – | 0/6 |

[*] Observed for 7 days.
[**] Control consisted of toxin administration only, no treatment.

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II.  SUBACUTE TOXICITY AND IMMUNOTOXICITY FOLLOWING INHALATION AND DERMAL

EXPOSURE OF PIGS TO T-2 TOXIN--Victor F. Pang

A.  Dermal Studies

INTRODUCTION AND OBJECTIVES

Interest in the group of trichothecene mycotoxins of particularly T-2 toxin, has been greatly aroused as accumulating evidence points towards their use in biochemical warfare by Soviet-supplied forces in Southeast Asia and elsewhere. Dermal exposure is one of the most direct and important routes of exposure. It has been shown that the permeability properties of skin and the general physiology, and the cardiovascular system of pigs are comparable to those of man (Reofenrath, et al.). Swine, therefore, were chosen as a suitable model for the study of dermal exposure to T-2 toxin.

The objectives of this study were: 1) to characterize the sequential morphologic changes of skin and other organs after a single sublethal topical dose of T-2 toxin, 2) to assess the possible effects of topically applied T-2 toxin on the immune system, hematologic parameters, serum enzymes and biochemistries, body temperature, and body weight.

METHODS AND RESULTS

Preliminary Studies

Two preliminary studies were carried out in order to determine an appropriate sublethal, but toxic dose, to establish procedural protocols and to evaluate experimental techniques of topical application and immunoassays for use in the formal study.

Two pigs were used in the first preliminary study. Sheep red blood cells (SRBC), two doses 21 days apart, were administered subcutaneously in 2 pigs. The response to this immunization was measured by lymphocyte transformation assays and hemagglutination titers. Adequate immunization was achieved by this route. For dosage determination, T-2 toxin was dissolved in 0.75 ml. of

dimethyl sulfoxide (DMSO) and applied at doses of 20 mg/kg and 15 mg/kg to one pig, each. The dissolved T-2 toxin was applied topically to a shaved area on the back, approximately 15 x 10 sq. cm. A protective non-occlusive foam pad device was used for preventing loss of the toxin, but leaving the dosed area indirectly exposed to the atmosphere through a layer of gauze. The 15 mg/kg dose proved to be sublethal, causing severe ulceration at the site of application with relatively good recovery of the animal after three weeks. The pig dermally dosed with 15 mg/kg T-2 toxin was immunized subcutaneously with SRBC at the same time of dosing followed by a second immunization 21 days later. Data obtained from the lymphocyte transformation assays in this single pig showed poor responses of the peripheral blood lymphocytes to both phytohemagglutinin (PHA) and concanavalin A (Con A), but possible increases in the lipopolysaccharide (LPS) and pokeweed mitogen (PWM) responses. Hemagglutination tests during the period of the primary immune responses revealed that the titers of anti-SRBC antibody (Ab) were slightly higher in treated than in untreated pigs.

Four pigs were used in the second preliminary study. T-2 toxin was applied topically at 10 mg/kg (2 pigs) and 15 mg/kg (2 pigs) in order to evaluate survival, clinical signs and possible immunologic effects. All pigs survived in this trial. Pigs exposed to T-2 toxin at 15 mg/kg had more severe clinical signs (fever, anorexia, lethargy) than pigs dosed at 10 mg/kg. No immunologic effects were seen in pigs dosed at 10 mg/kg. Effects on the immune system of the pigs dosed at 15 mg/kg were similar to those seen in the pig used in the first preliminary study, although individual variation was evident.

FORMAL STUDIES

1) **Histopathological effects of subacute dermal exposure of T-2 toxin**

In this study, histopathologic effects of dermally applied T-2 toxin were evaluated in 26 crossbred, female, specific-pathogen-free (SPF) pigs. T-2 toxin was dissolved in 0.75 ml DMSO and applied topically as described in the preliminary studies at doses of 0 (8 control pigs) and 15 mg/kg (18 pigs). Animals were killed sequentially on 1, 3, 7, and 14 days postdosing. A complete necropsy was performed and representative tissue samples were fixed for histopathologic examination.

Grossly, the areas of skin exposed to T-2 toxin were swollen, and dark red on days 1 (Fig. IIA1) and 3 (Fig. IIA2), and became purple and dark purple on day 7 (Fig. IIA3) and day 14 (Fig. IIA4), respectively. Prominent scale formation was noted on day 3 and became progressively worse on days 7 and 14. On day 7, the affected skin was ulcerated with serosanguinous exudation, especially at the margin of the exposed regions. These lesions became bloody and were covered by a thick, crusty scab on day 14. The affected skin and the underlying tissue was focally separated at the border of the exposed area.

On day 1, the histologic changes were located primarily in the epidermis and upper dermis, although occasional mild involvement of lower dermis and superficial subcuticular fat was present. The lower dermis and subcuticular fat were affected in all pigs on and after day 3. The severity and depth of the lesions in the 3 layers of skin increased progressively from day 1 to day 14. The changes in the epidermis on day 1 were characterized by multifocal mild to moderate ballooning, degeneration and cellular dissociation (possibly due to intercellular edema) of epithelial cells of the strata spinosum and basale, along with formation of vesicles and mild infiltration by neutrophils and eosinophils (Fig IIA5). Some of the infiltrating inflammatory cells were

undergoing degeneration and necrosis. In addition to more prominent balloning degeneration, cellular dissociation, and vesicular formation, severe epithelial necrosis, marked neutrophilic infiltration, and formation of multifocal intraepidermal abscesses were present in the mildly to moderately acanthotic epidermis on day 3 (Fig. IIA6). In some of the severely involved regions, suprabasilar intraepidermal bullae and clefts were also observed. Locally extensive necrosis and subcorneal accumulations of neutrophils accompanied by marked parakeratosis and pseudoepitheliomatous hyperplasia were noted on days 7 and 14 (Fig. IIA7), but ballooning degeneration and vesicular formation had become less evident than on day 3.

The dermis on day 1 was infiltrated, mainly around the vessels, by a moderate number of neutrophils admixed with some eosinophils. Degeneration and necrosis were seen in many of the inflammatory cells. Mild edema, fibrin deposition, and disruption of collagen were present in the papillary layer and perivascular regions of the upper reticular layer. Fibrinoid degeneration and formation of fibrin thrombi occurred in some of the capillaries of the upper dermis. These changes, especially the infiltration of inflammatory cells, became more diffuse and prominent on day 3. The infiltrate was a mixture of macrophages, lymphocytes, eosinophils and especially neutrophils. Degeneration and necrosis of the inflammatory cells were evident in the upper dermis. Small perivascular cuffs of mixed inflammatory cells with mild perivascular edema and fibrin deposition were constantly observed in the superficial subcuticular fat. In addition to an increase in intensity of the changes occurring in the dermis and subcutis on day 3, mild to moderate fibroplasia began to appear on day 7. Although fibroplasia became more evident, extensive necrosis was still present in the derr'; on day 14. Hyperplasia with

papillary folding of the lining epithelium of sweat glands in the lower dermis was seen more often on day 14 than on day 7.

The changes in the internal organs were minimal. Slight to mild increases in the number of tangible body macrophages was found in the cortex of the thymus. Occasional single cell necrosis was noted in the follicles of lymphoid organs and tissues. There were scattered single cell vacuolization and necrosis in the acinar cells of the pancreas. These changes were randomly present on all four days, although they were more often seen on days 1 and 3.

This study demonstrated that T-2 toxin has a profound, direct, cytotoxic effect on skin. Based on the occurrence and severity of lesions, it appears that the cells of stratum germinativum (including strata basale and spinosum), especially the basal layer, were the main target of T-2 toxin. This can be explained by the fact that cells of this layer are highly metabolically active, which involves both protein and DNA synthesis, and that T-2 toxin has been well documented to be a strong inhibitor of protein synthesis with subsequent inhibition of RNA and DNA synthesis. Endothelial cells might be another target of T-2 toxin, because vascu .tis and formation of microthrombi were also evident on day 1. The extensive necrotizing dermatitis that subsequently developed might have been a result of augmentation by significant amounts of lysosomal enzymes released from the large numbers of degenerating and necrotic inflammatory cells, mainly neutrophils and macrophages.

Although the healing process was recognized on day 7, the necrotizing dermatitis persisted during the first 2 weeks postdosing. Significant quantities of T-2 toxin and several metabolites were detected from the skin and subcuticular fat of exposed and nearby regions throughout the study (see Section V). It has been suggested that dermis may provide a significant

barrier or reservoir for lipid soluble compounds (Reofenrath, et al.).
Therefore, it is speculated that skin may act as a reservoir of T-2 toxin from
which a small amount of the toxin and its metabolites are constantly released
into the blood stream. This may also explain why dramatic damage occurred in
the dosed skin, but the histologic changes in the internal organs were
minimal.

2. Subacute toxicity of topically applied T-2 toxin on the immune
system, hematology, serum enzymes and biochemistries, body temperature, and
body weight.

The effects of a single, sublethal dose of topically applied T-2 toxin on
the immune system, hematology, serum enzymes and biochemistries, body
temperature, and body weight were evaluated in 14 crossbred, castrated, male,
SPF pigs. T-2 toxin was applied topically as described previously at doses of
0 (6 control pigs) and 15 mg/kg (8 test pigs) on day 0. The pigs were
immunized subcutaneously with sheep red blood cells (SRBC) at the time of
dosing and 21 days later. Blood was collected from the anterior vena cava on
days -2, 1, 3, 5, 7, 10, 14, 20, 22, 24, 26, 28, and 31 for hematology and
immunology studies. Serum samples during the first 2 weeks were used for
analysis of serum proteins, enzymes, glucose, cholesterol and electrolytes.
Body temperature was measured daily during the first 2 weeks and on days 16,
18, 21, 23, 27, and 30. Pigs were weighed before dosing and weekly postdosing
for 5 weeks. The split-plot design with repeated measurements was used as the
statistical model for analyzing the data. The analysis was done with the
Statistical Analysis System (SAS) statistical package. Differences were
considered statistically significant when P-values were less than 0.05.

IMMUNOLOGY STUDY

In this study, the effects of T-2 toxin on the immune system were evaluated at the cellular level with lymphocyte transformation assays. Using 4 different mitogens (LPS, PHA, Con A, and PWM) on purified peripheral blood lymphocytes. Responses were also evaluated by the quantitation of Ab production in the hemagglutination test.

The ability of swine peripheral blood lymphocytes to respond to mitogens was measured by their ability to incorporate $^3$H thymidine. The data was compared in two different ways as mean counts per minute (M CPM) where M CPM = (mitogen-stimulated CPM)/number of pigs in each group, and mean stimulation index (MSI) where MSI = (mitogen-stimulated CPM/background CPM)/number of pigs in each group.

1) Responses to Con A - At a concentration of 50 ug/ml (optimal concentration), significantly lower responses were found in the test group at day 22 in M CPM and at days 22, 26, and 28 in MSI. No significant differences were found at a concentration of 10 ug/ml (suboptimal concentration) in either M CPM or MSI (Fig. IIA8, IIA9).

2) Responses to PHA - At a concentration of 20 ug/ml (optimal concentration), significantly lower responses were found in the test group on days 5, 22, and 26 in M CPM and on days 20, 22, 24, 26, and 28 in MSI. No significant differences were found at a concentration of 2 ug/ml (suboptimal concentration) in both either M CPM or MSI (Fig. IIA10, IIA11).

3) Responses to LPS - At a concentration of 2 ug/ml (suboptimal concentration), significantly higher responses were found in the test group on days 3, 5, 7, and 10 in M CPM and on day 14 in MSI. At the optimal concentration, 20 ug/ml, a significantly higher response was found in the test

group only on day 10 in M CPM, but no significant differences were seen in MSI (Fig. IIA12, IIA13).

4) Responses to PWM - At an optimal concentration of 1:200, significantly lower responses were noted in the test group on day 5 in M CPM and on days 22 and 26 in MSI. Significantly higher responses were present in the test group on day 5, 3 and 7 in M CPM. At a suboptimal concentration of 1:400, significantly lower responses were found on day 5 in M CPM and on day 22 in MSI (Fig. IIA14, IIA15).

Lymphocyte transformation assays are useful for assessing the gross immunological competence of humans and other animals. Lectin-induced mitogenesis can involve macrophages, T-lymphocytes and B-lymphocytes. The lectins that were used in this study are somewhat lymphocyte-specific. For example, Con A is a broad T-cell mitogen, PHA is mostly a T-effector cell mitogen, PWM is a mitogen activating both Band T-cells, and LPS is a B-cell mitogen. As illustrated above, the study showed that a single, topically applied, sublethal dose (15 mg/kg) of T-2 toxin in pigs could cause significantly lower responses of peripheral blood lymphocytes to mitogens, PHA, Con A, and possibly, PWM. Although no significant morphological changes were observed in the lymphoid system in the previous histopathological study, it is reasonable to speculate that T-2 toxin might cause functional depression on T-cell populations. Most of these significant reductions in T-cell function occurred between days 20 and 28. Based on these findings, and the results of dermal residue analyses for T-2 toxin and metabolites, it appears that the functional depression probably was due to cumulative effects of constantly released T-2 toxin and metabolites from this dermal reservoir. This functional depression was reversible. Conversely, significantly higher

responses to the B-cell mitogen LPS (at least in M CPM) occurred mainly within the first 2 weeks postdosing.

The ability of T-2 dosed animals to produce antibodies was measured by the titers of anti-SRBC Ab with hemagglutination tests. Although the mean titers of anti-SRBC Ab of the test group were approximately 2-fold higher than those of the control group on days 10, 14, 22, and 24, no statistically significant difference were present at these time points (Fig. IIA16). SRBC are T-cell dependent immunogens. Anti-SRBC Ab production is controlled by helper T-cells and suppressor T-cells, although macrophages are also important by presenting antigenic determinants of SRBC to both T-cells and B-cells. According to the data from both lymphocyte transformation assays and hemagglutination tests, it appears that topically applied T-2 toxin in pigs had no effects on the specific helper and suppressor T-cells, or on the ability of B-cells to produce anti-SRBC Ab. Thus, the effects on these cells were not sufficient to significantly influence the Ab production.

Aside from helper and suppressor cells, T-cells contain at least two more populations, the cytotoxic and delayed type hypersensitivity T-cells. Although Ab is an important arm of the immune system against many infectious agents, without the coordination of cellular immunity, it often is insufficient to provide full protection for the host. It is well known that cellular immunity plays a very important role in viral, intracellular bacterial, protozoal infections, and in tumor cell killing. Therefore, the functional suppression of T-cells shown in this study might be of importance in disease defense, in spite of absence of effects on anti-SRBC Ab production. This is an important area which needs further elucidation.

CLINICAL PATHOLOGY STUDY

1) Hematology

The mean total white blood cell count of the test group was significantly higher in the first 2 weeks postdosing (Fig. IIA17). It increased gradually after dosing and reached a peak at day 10 and returned to normal at day 20. The leukocytosis was mainly due to an absolute increase in neutrophils (Fig. IIA18) and also due to an apparent increase in the absolute number of monocytes on days 3 and 14 (Fig. IIA19). The neutrophilia was coincided with the severe necrotizing dermatitis induced by T-2 toxin. Before dosing, the mean total lymphocyte count of the test group was significantly higher than that of the control group, but it was significantly lower on post-dosing on days 7 and 14, (Fig. IIA20).

The mean total erythrocyte count of the test group was significantly lower than that of the control group on days 1, 3, and 5 (Fig. IIA21). It dropped sharply on day 1, then gradually increased, but was still significantly depressed on days 3 and 5. No differences were present after day 5. Both mean hemoglobin and mean packed cell volume of the test group were significantly lower than those of the control group on days 10 and 14 Fig. IIA22, IIA23).

2) Serum Proteins, Enzymes, Glucose, Cholesterol, and Electrolytes

The mean total serum protein of the test group was significantly higher than that of the control group on days 5 and 7 (Fig. IIA24). The mean serum albumin of the test group was significantly lower than that of the control group on days 7, 10, and 14 (Fig. IIA25). The difference started on day 3, but was not significant until day 7, and became gradually more prominent. Anorexia of treated pigs during the first week might play an important role. Conversely, the mean serum globulin of the test group gradually increased

after dosing, became significantly higher than that of the control group on day 5, and reached a plateau after day 7 (Fig. IIA26). The source of the increased globulin is uncertain, but it could be (at least partially) inflammatory. Significantly lower mean serum alkaline phosphatase activities were present in the test group on days 3, 5, 7, 10, and 14 (Fig. IIA27). This decline and that of serum albumin might possibly be related to decreased protein synthesis, caused by T-2 toxin directly. No significant differences were noted in SGOT, SGPT, and LDH.

The mean blood glucose of the test group dropped markedly from day 1. Although the reduction eventually abated, the concentration was significantly lower than that of the control group throughout the first 2 weeks (Fig. IIA28). A significantly lower mean serum cholesterol concentration was present on days 1, 3, and 10 in the test group. This might also be associated with decreased feed uptake.

The mean serum phoshorus concentration of the test group was significantly lower than that of the control group on days 3, 7, and 14. In addition to these 3 days, the mean serum calcium was also significantly reduced on day 10. No differences were noted in sodium, chloride or potassium.

3) Body Temperature and Body Weight

A significant increase in mean rectal temperature of the test group, as compared to the control group, occurred during the first 2 weeks, but differences were not significant at other points (Fig. IIA29). Neutrophils and macrophages are the two main sources of intrinsic pyrogens and probably served as sources in this study.

The mean body weights of the test and control groups were about the same on the day of dosing. The rate of gain of the test group was lower than that of the control group throughout the entire study. Significant differences were seen at weeks 2, 3, 4, and 5 (Fig. IIA30).

Fig. IIA1   The area of skin exposed tc T-2 toxin ( 5 mg/kg) of a pig killed on day 1.  It shows swelling and dark red discoloration.

Fig.  IIA2   The area of skin exposed  to  T-2 toxin (15 mg/kg) of a pig killed on day 3.  It shows marked swelling and purple discoloration  with scale formation.

1

2

Fig. IIA3   The area of skin exposed to T-2 toxin (15 mg/kg) of a pig killed on day 7.   It shows dark purple discoloration and ulcers with serosanguinous exudation, mainly at the margin of the exposed region.

Fig. IIA4   The area of skin exposed to T-2 toxin (15 mg/kg) of a pig killed on day 14.   It shows dark purple discoloration, ulcers with hemorrhage, formation of thick crusty scab and separation from the underlying tissue at the margin of the exposed region.

3

Fig.   IIA5   Skin of a pig killed on day 1. Multifocal mild to moderate ballooning degeneration and cellular dissociation of stratum germinatrium with formulation of vesicles and mild infiltration by neutrophils and eosinophil: in the epidermis. The dermis also has congestion, edema and small perivascular cuffs of neutrophils. HE stain, 560 x.

Fig.   IIA6   Skin of a pig killed on day 3. Extensive cellular necrosis and formation of suprabasilar intraepidermal clefts of the epidermis. There is severe diffuse inflammatory cell infiltration, edema, fibrin deposition and disruption of collagen of the dermis. HE stain, 560 x.
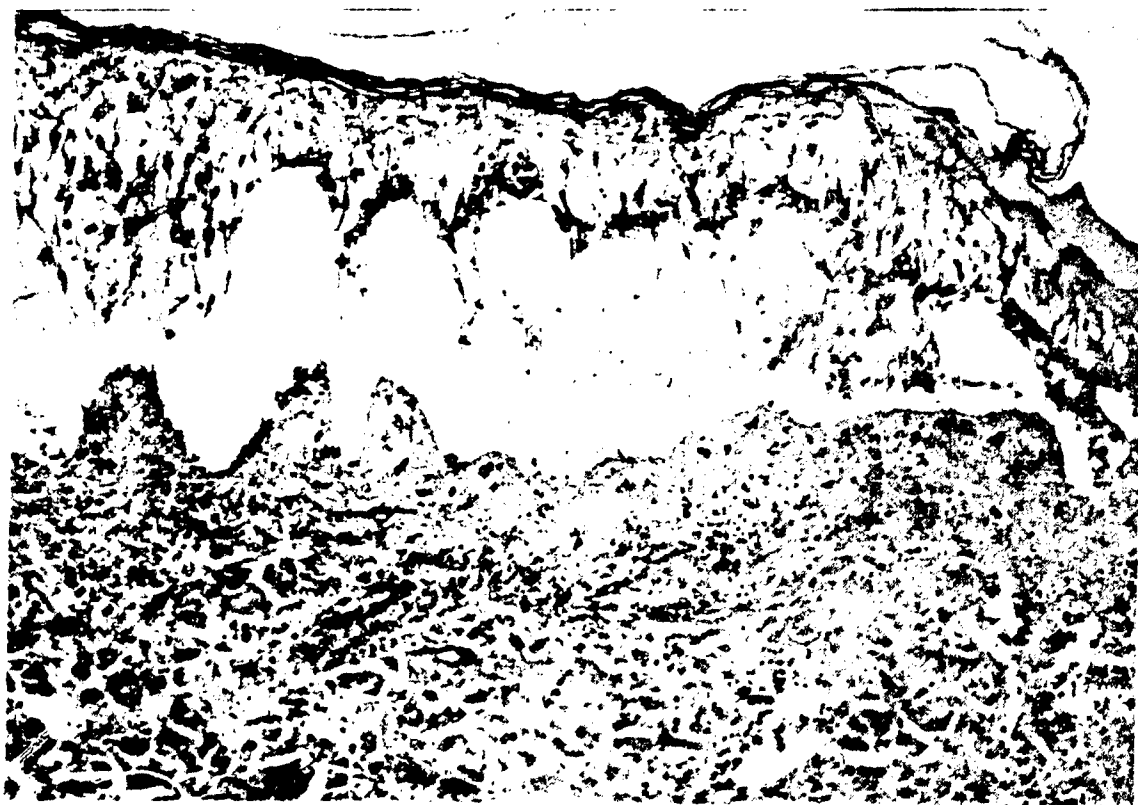
Fig. IIA7   Skin of a pig killed on day 7.  There is subcorneal accumulation
            of neutrophils and prominent pseudoepitheliomatous hyperplasia
            of the epidermis.  There is edema, fibroplasia and severe
            inflammatory cell infiltration of the dermis. HE stain, 225 x.
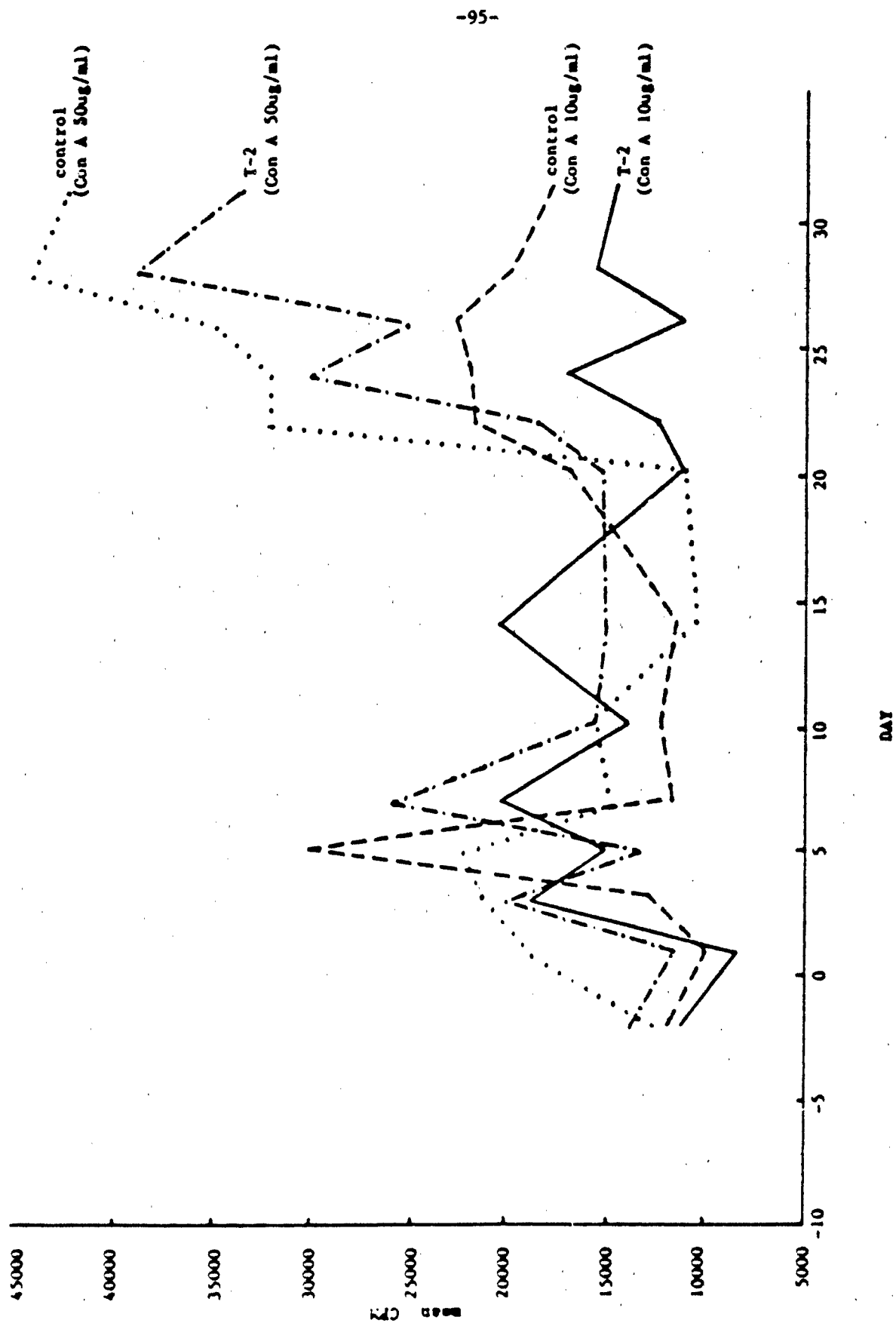
Fig. IIA8  Blastogenic responses (M CPM) of porcine peripheral blood
lymphocytes to the optimal (50 ug/ml) and suboptimal (10 ug/ml)
concentration of Con A of pigs exposed dermally to T-2 toxin
(15 mg/kg) and from vehicle exposed controls.
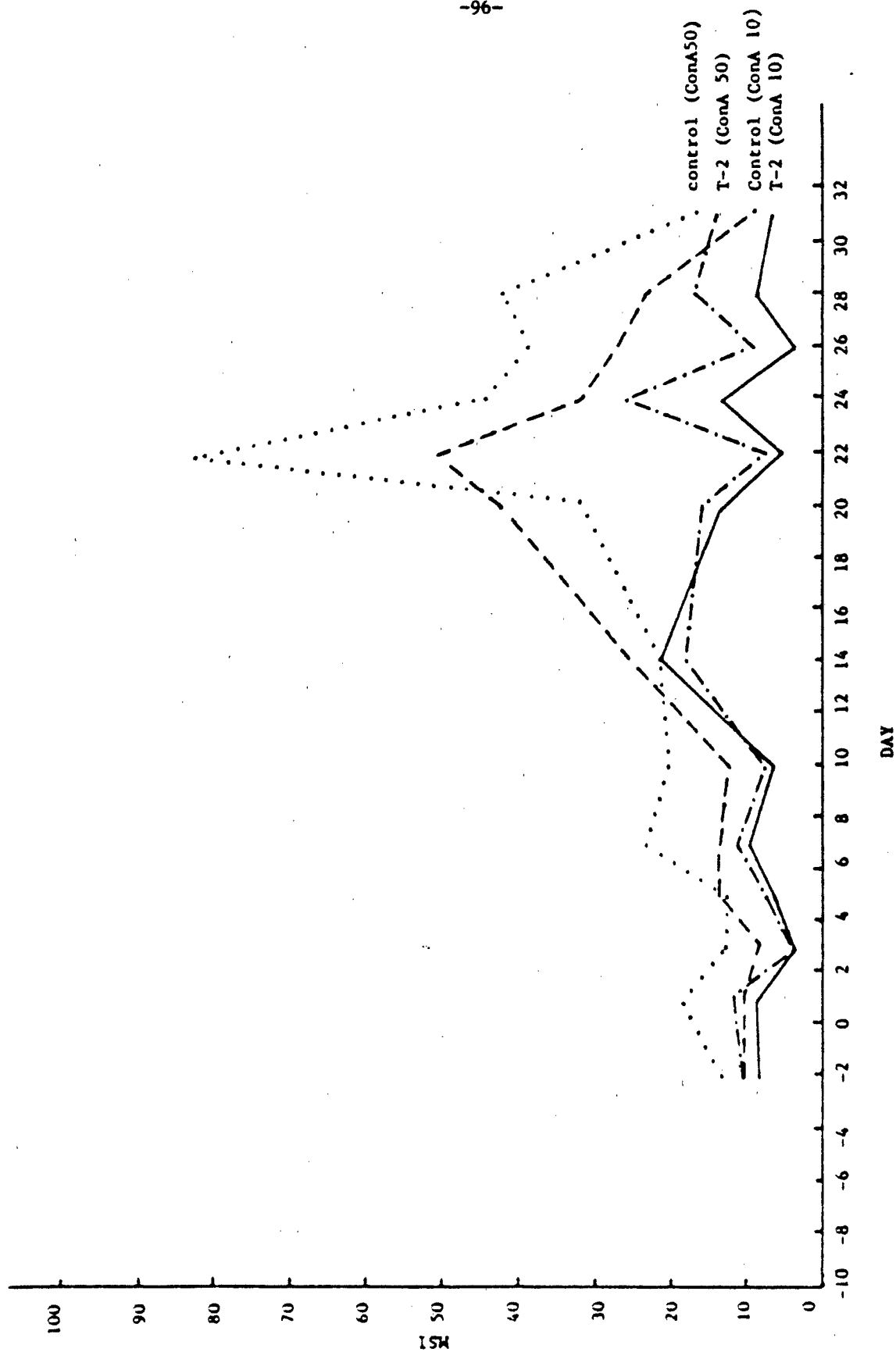
Fig. II.\9  Blastogenic responses (MSI) of porcine peripheral blood lymphocytes
to the optimal (50 ug/ml) and suboptimal (10 ug/ml) concentrations
of Con A of pigs exposed dermally to T-2 toxin (15 mg/kg) with
vehicle or to the vehicle alone.
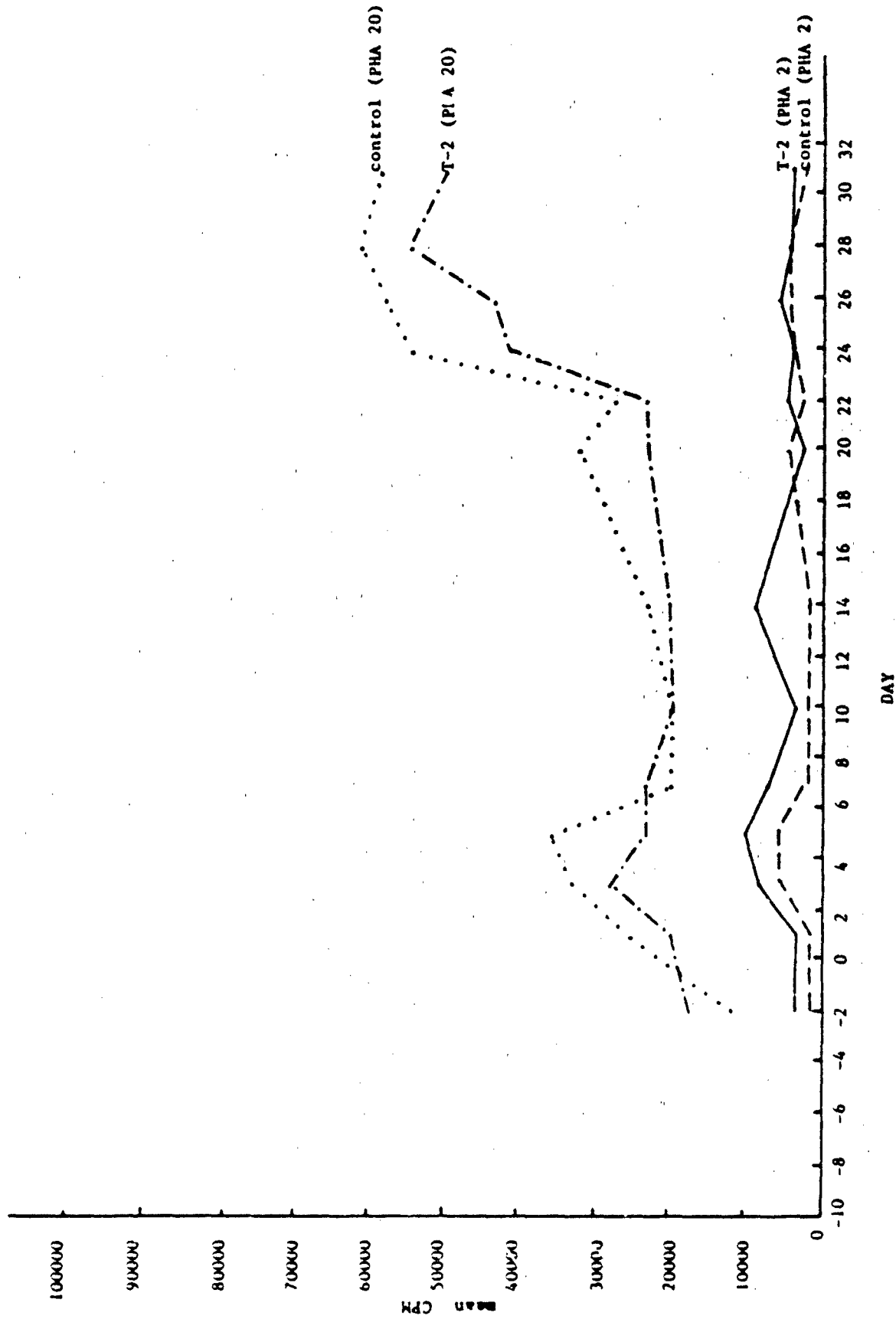
Fig. I1A10  Blastogenic responses (M CPM) of porcine peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentrations of PHA of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
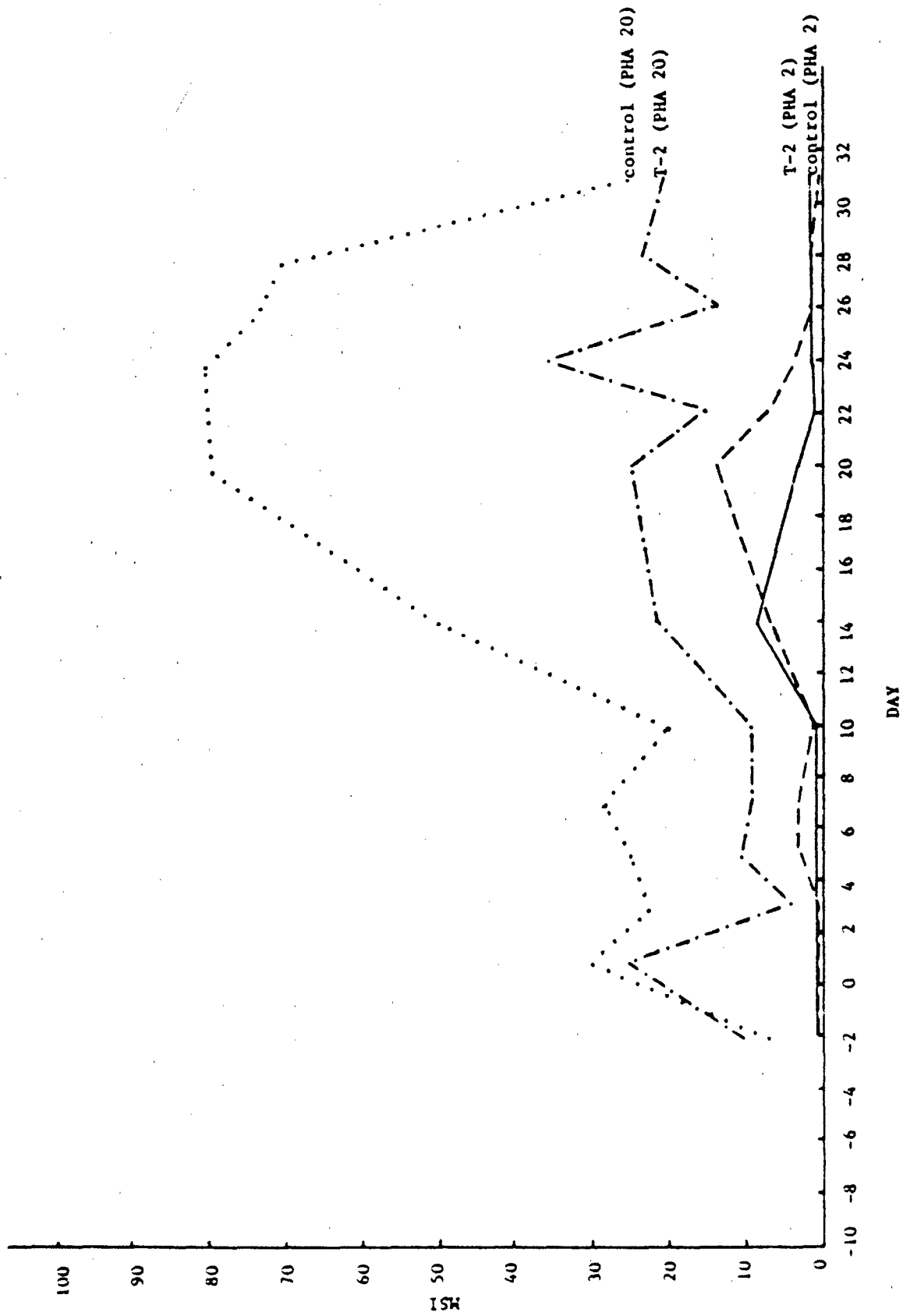
Fig. IIAII  Blastogenic responses (MSI) of porcine peripheral blood lymphocytes
to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentration
of PHA of pigs exposed dermally to T-2 toxin (15 mg/kg) with
vehicle and vehicle only.
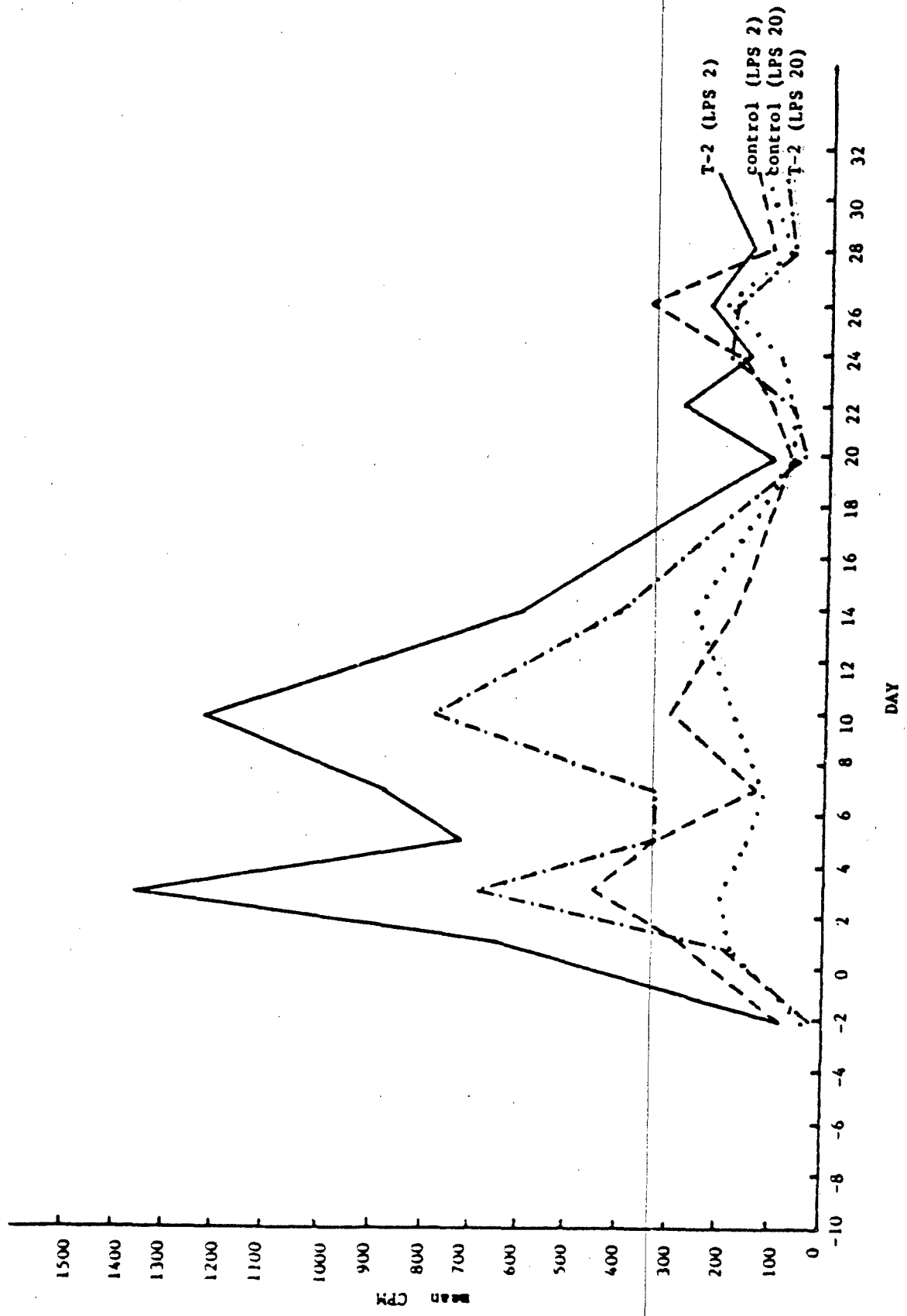
Fig. 11A12  Blastogenic responses (1₁ CPM) of porcine peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentration of LPS of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and vehicle only.
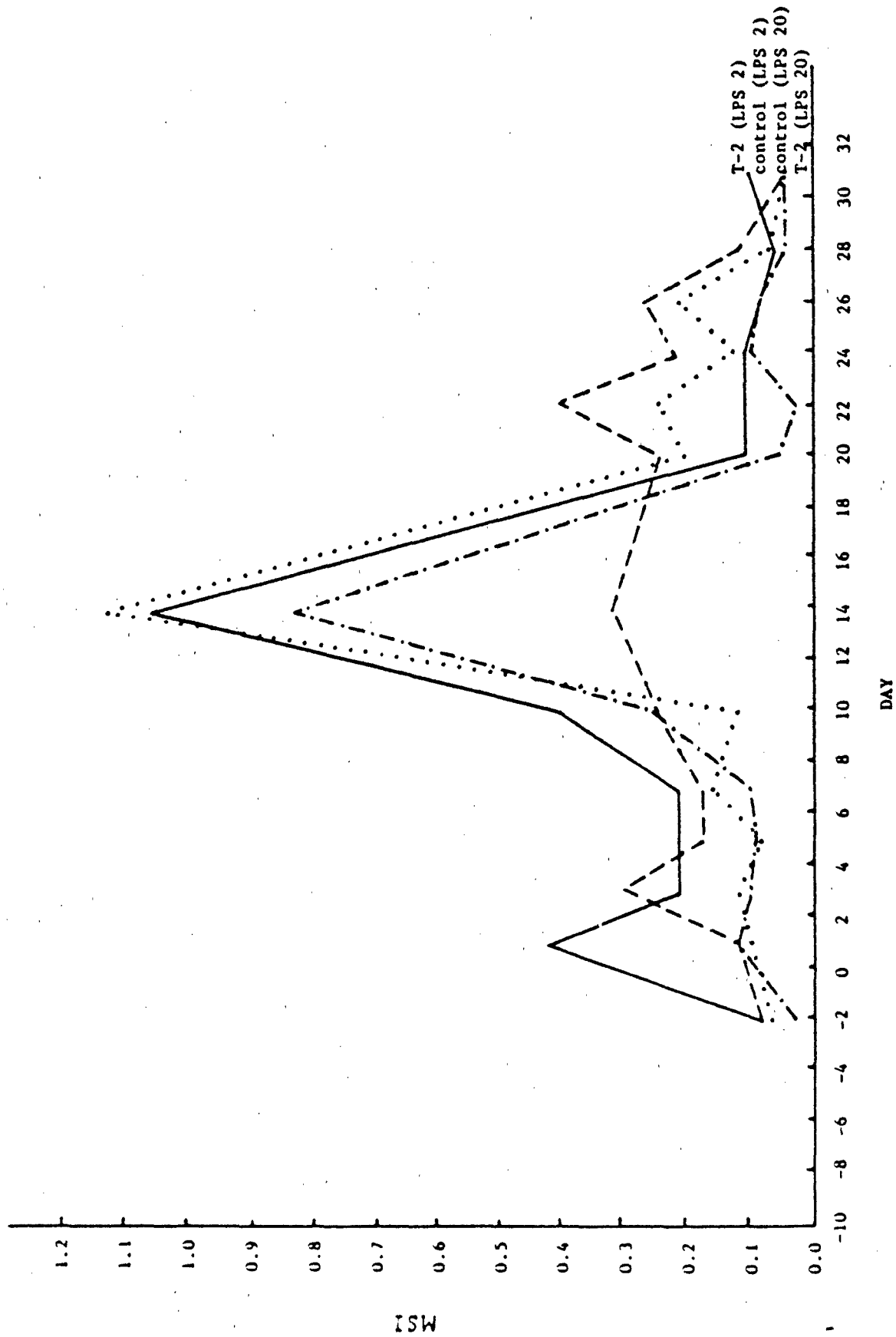
Fig. IIA13  Blastogenic responses (MSI) of porcine peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentration of LPS of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and vehicle only.
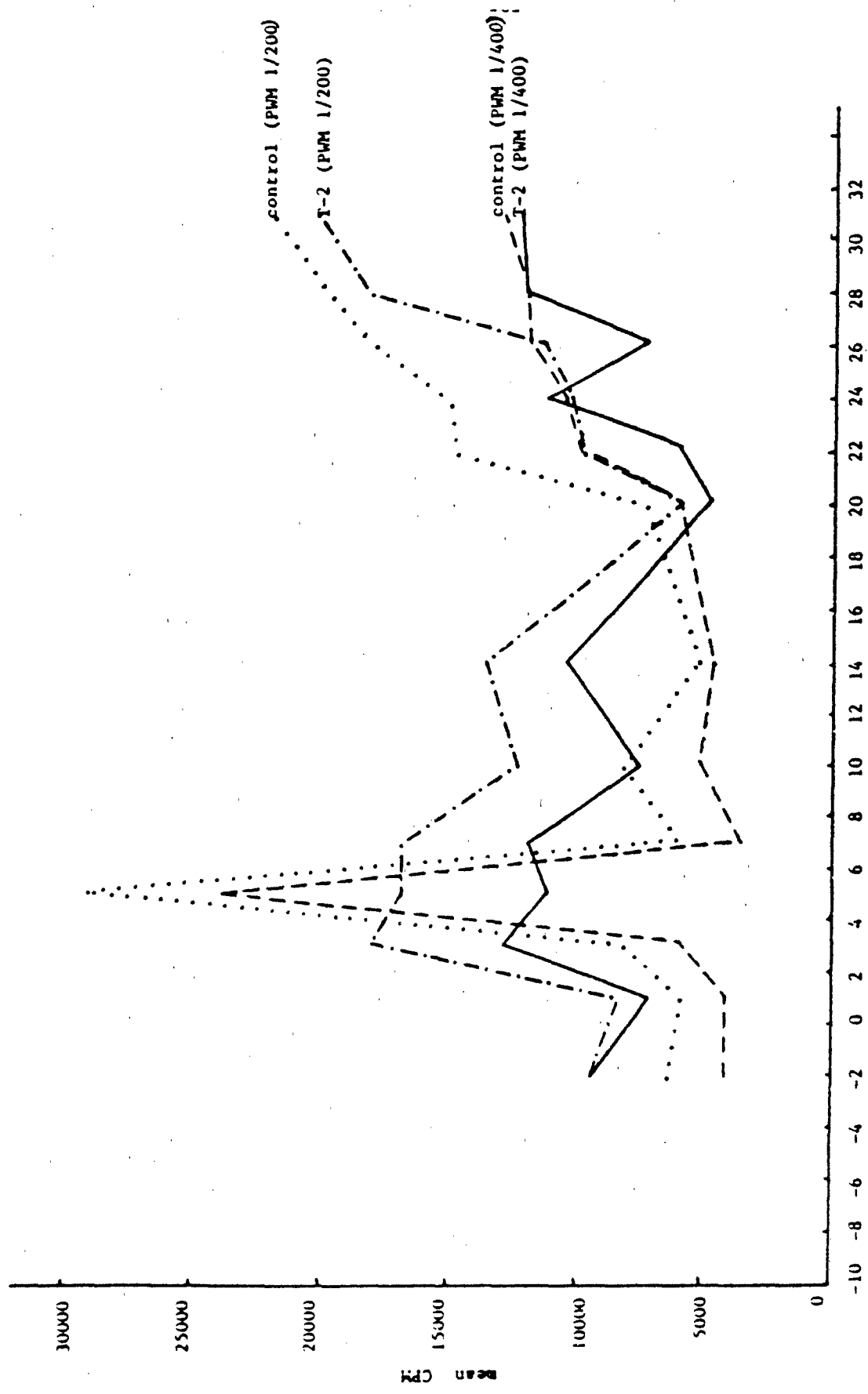
Fig. IIA14  Blastogenic responses (M CPM) of porcine peripheral blood lymphocytes to the optimal (1:200) and suboptimal (1:400) concentration of PWM of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and vehicle only.
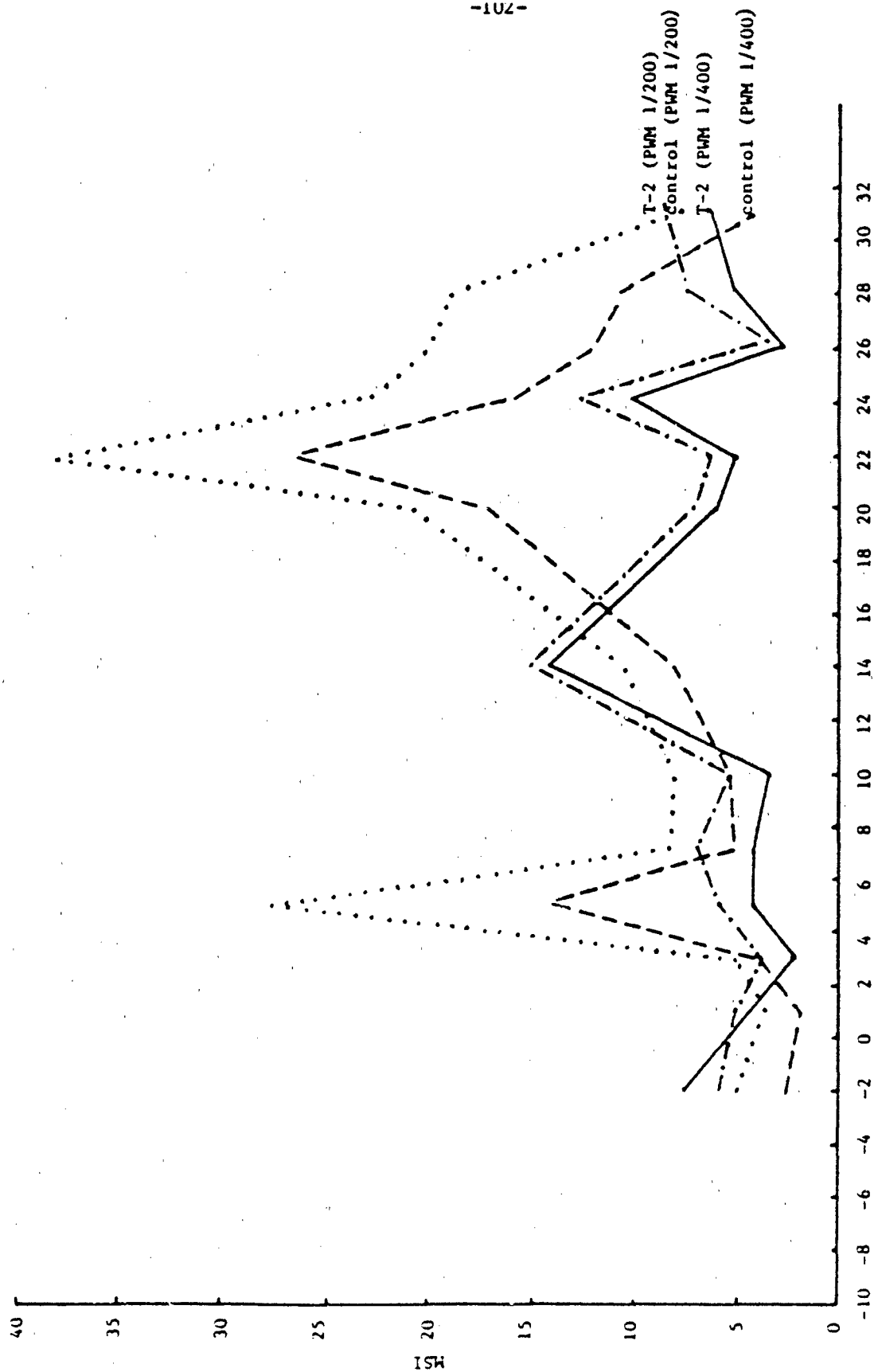
Fig. IIA15  Blastogenic responses (MSI) of porcine peripheral blood lymphocytes to the optimal (1:200) and suboptimal (1:400) concentrations of PWM of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and vehicle only.
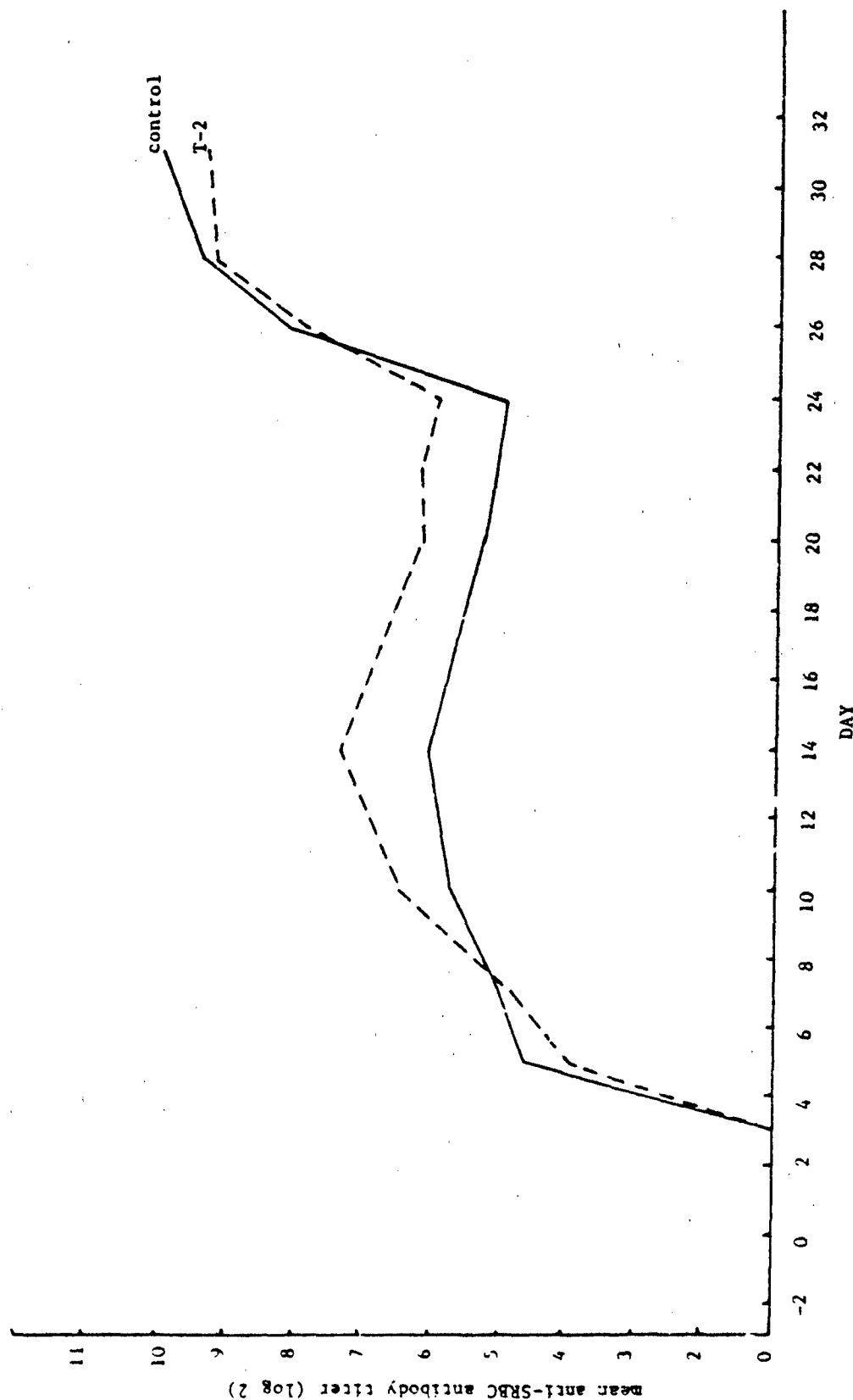
Fig. IIAl6 Mean anti-SRBC Ab titers of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and vehicle only.
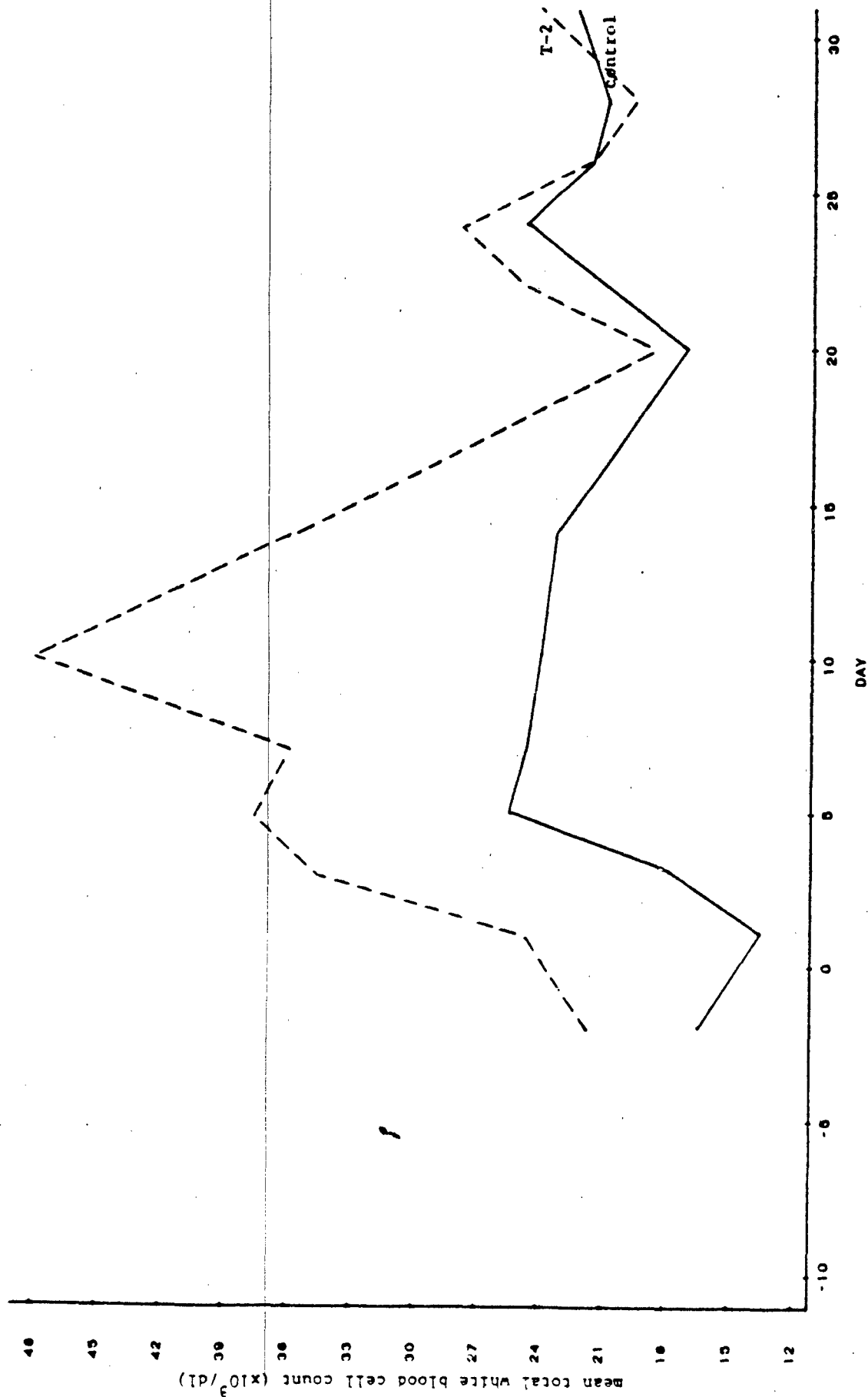
Fig. IIA17 Mean total white blood cell counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
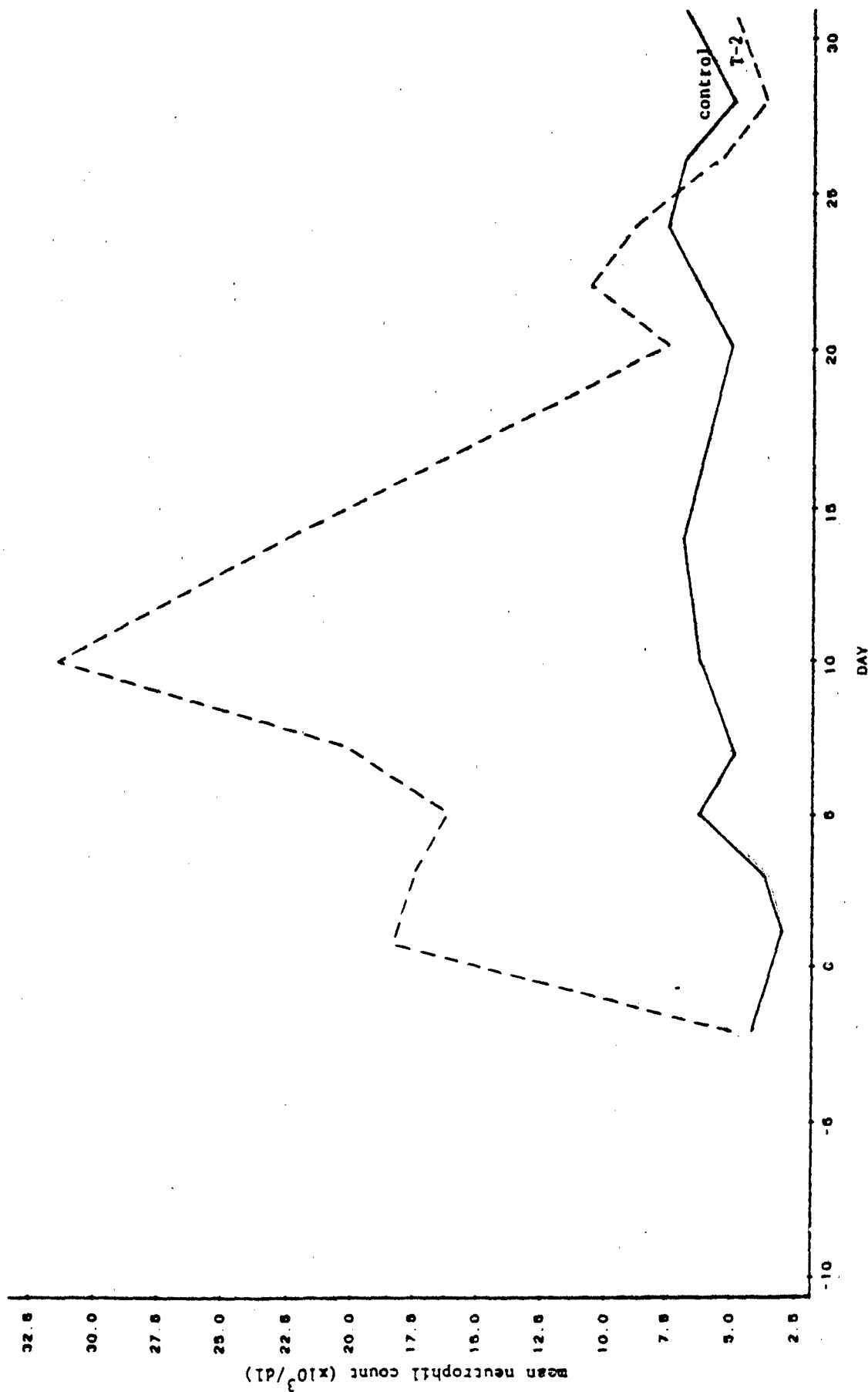
Fig. IIA18  Mean neutrophil counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
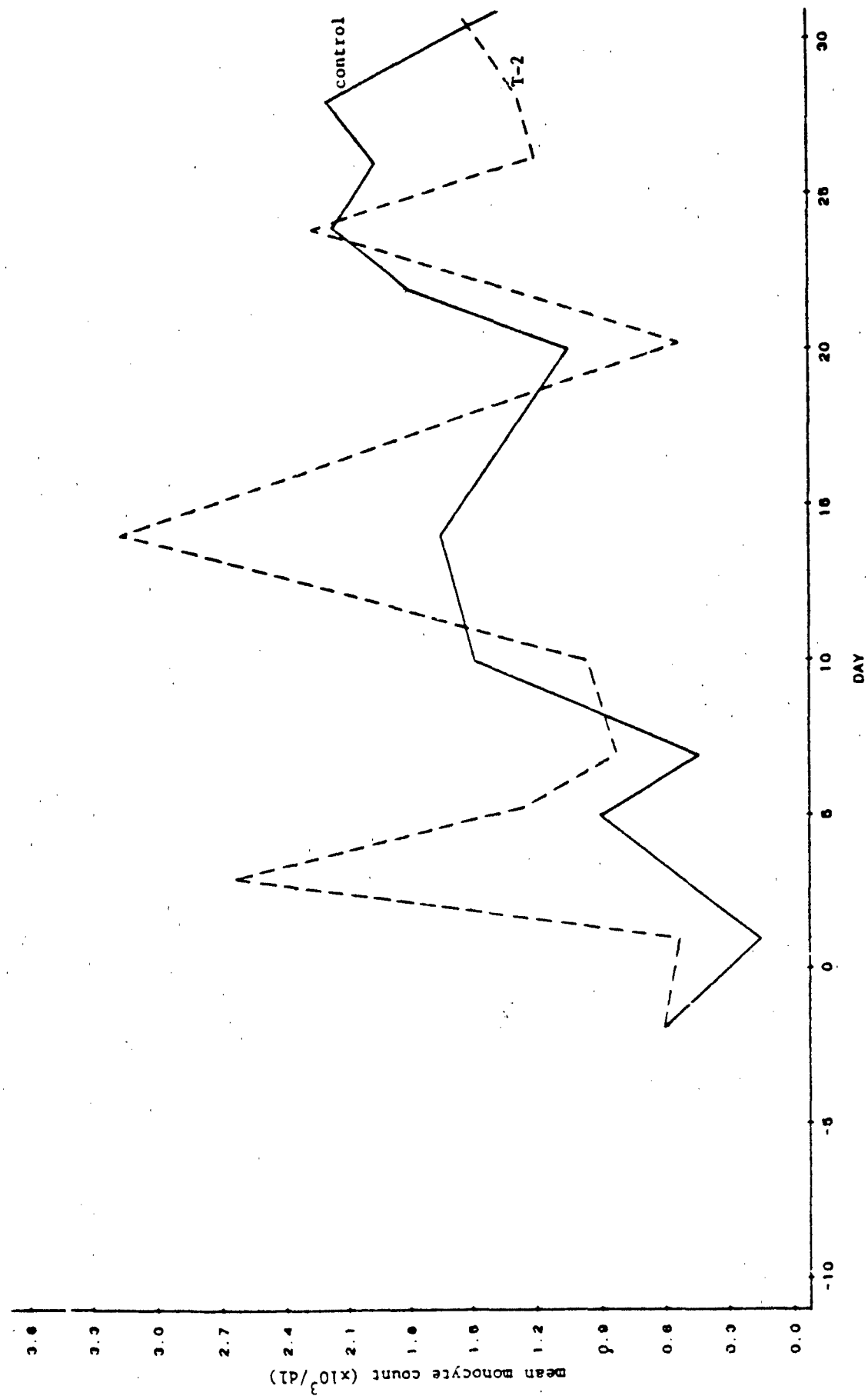
Fig. IIA19  Mean monocyte counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
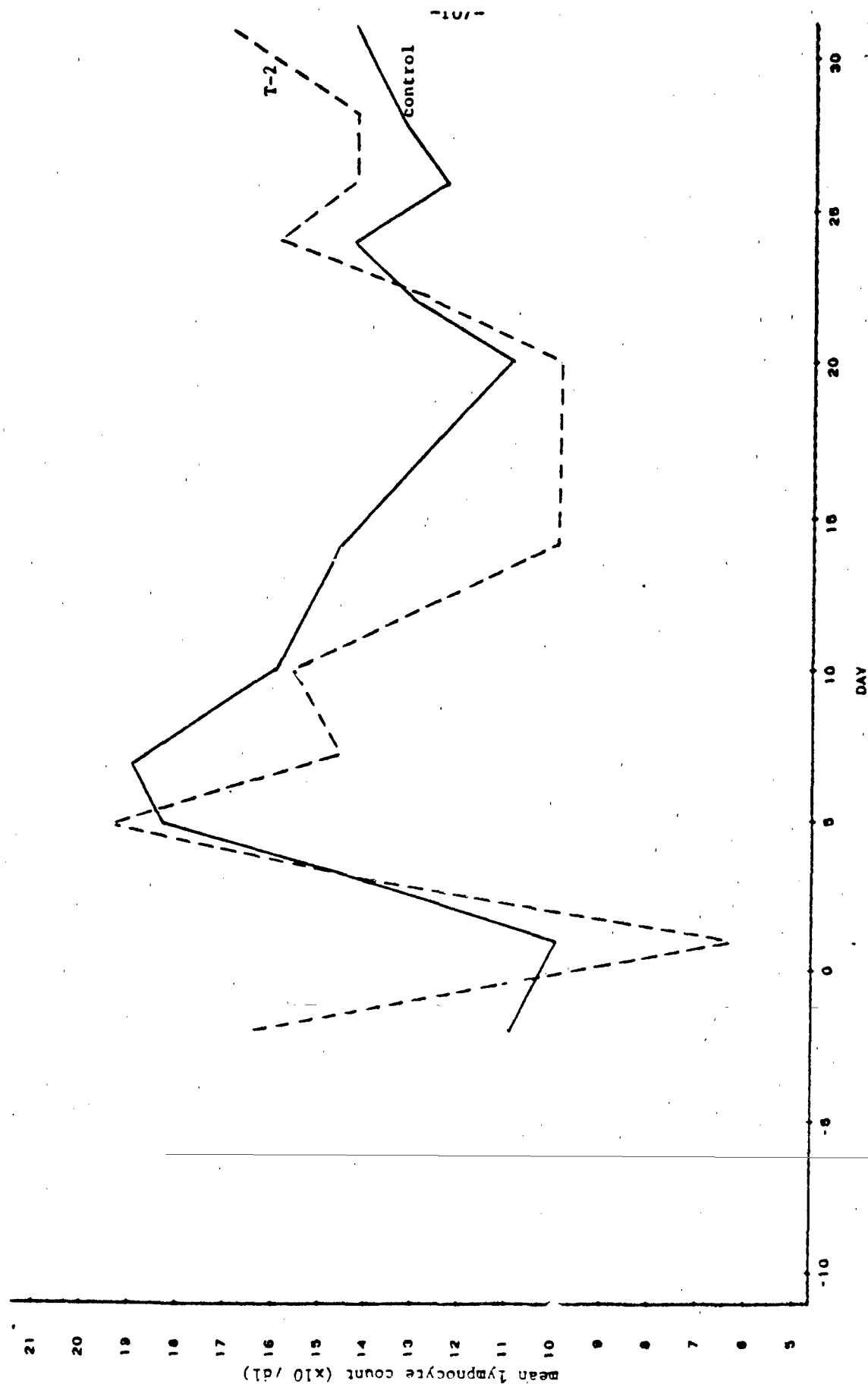
Fig. IIA20 Mean lymphocyte counts of pigs exposed dermally to T-2 toxin
(15 mg/kg) with vehicle and groups exposed to the vehicle only.
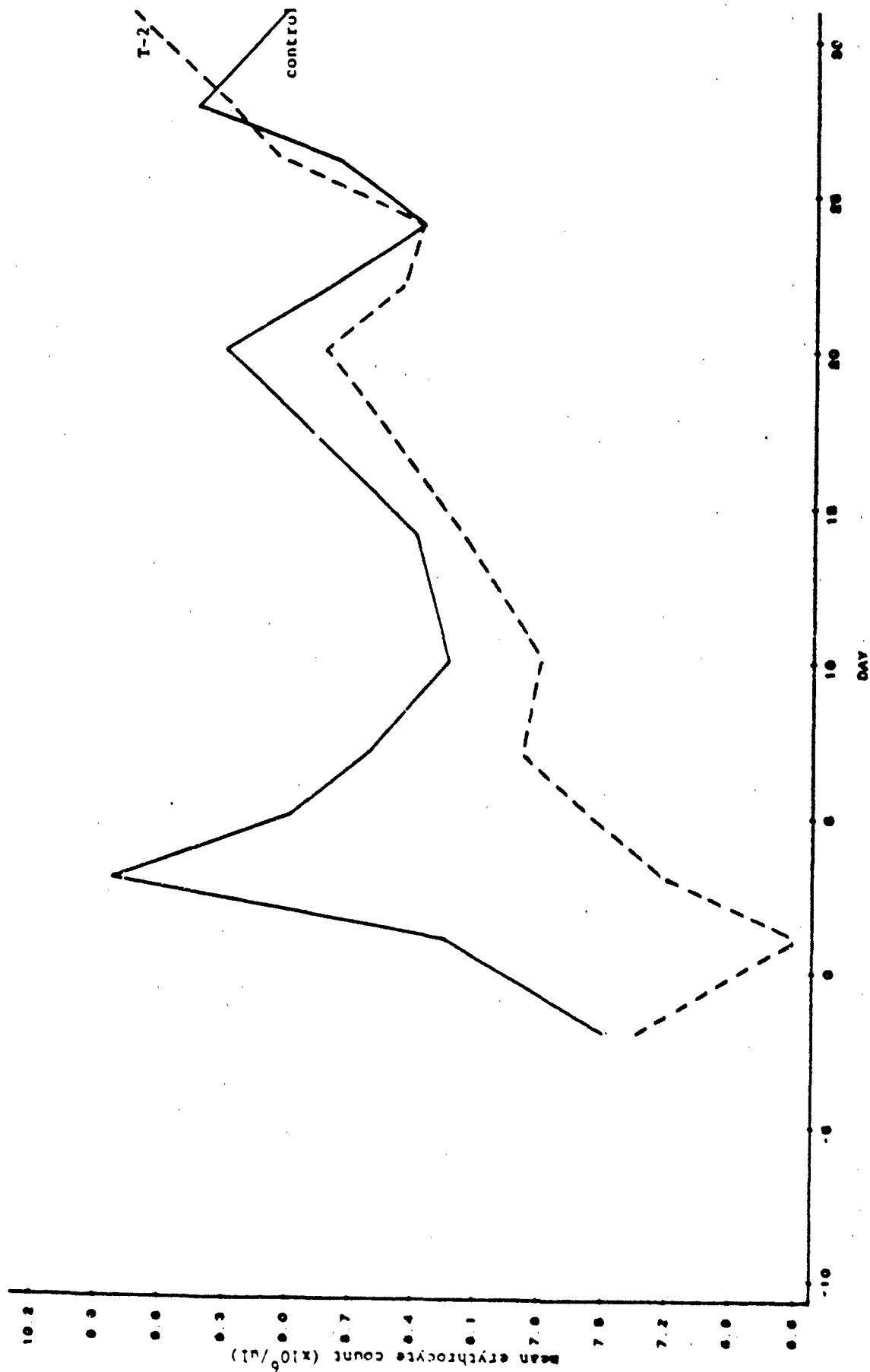
Fig. IIA21  Mean total erythrocyte counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
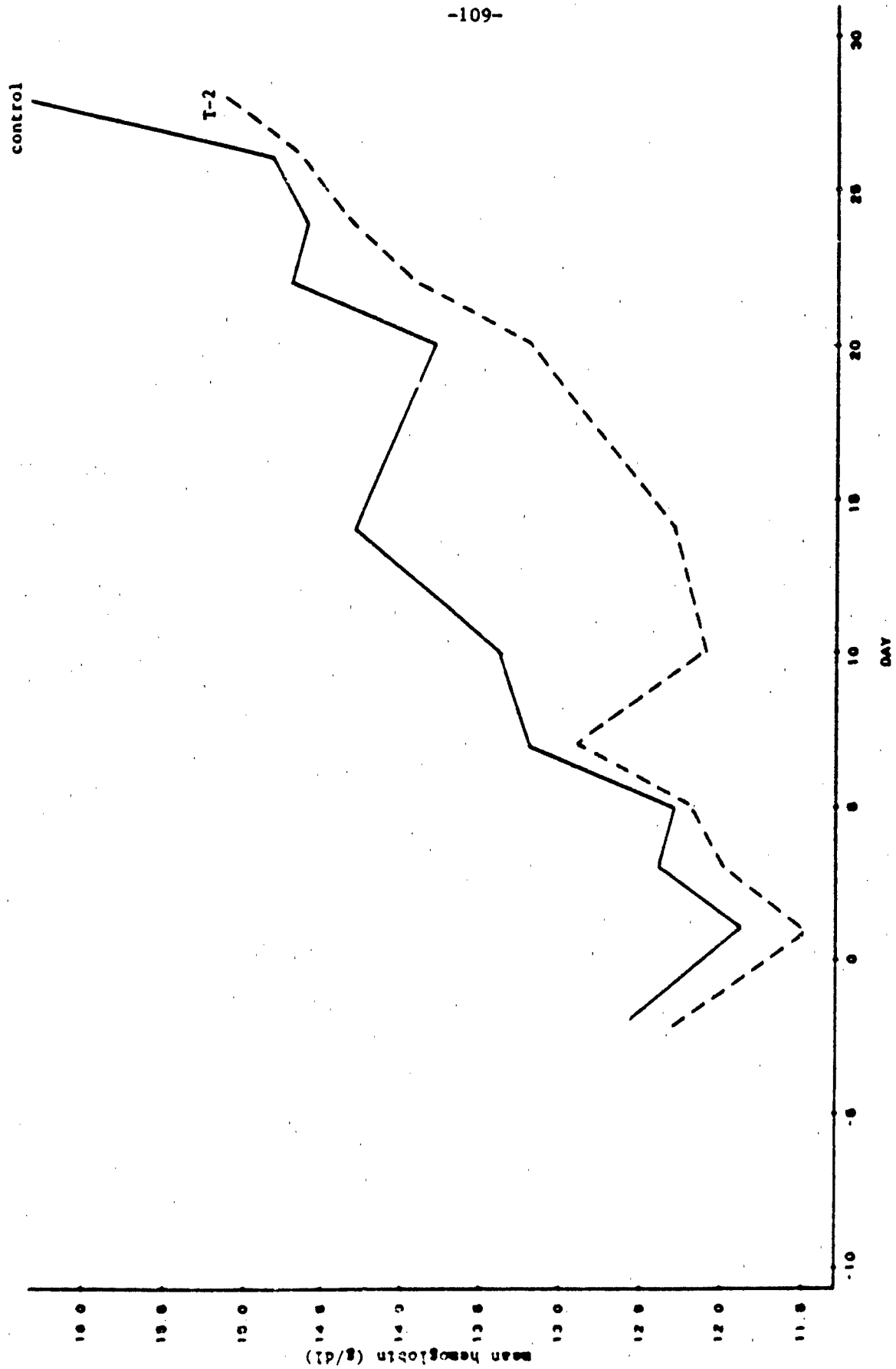
Fig. IIA22  Mean hemoglobin of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
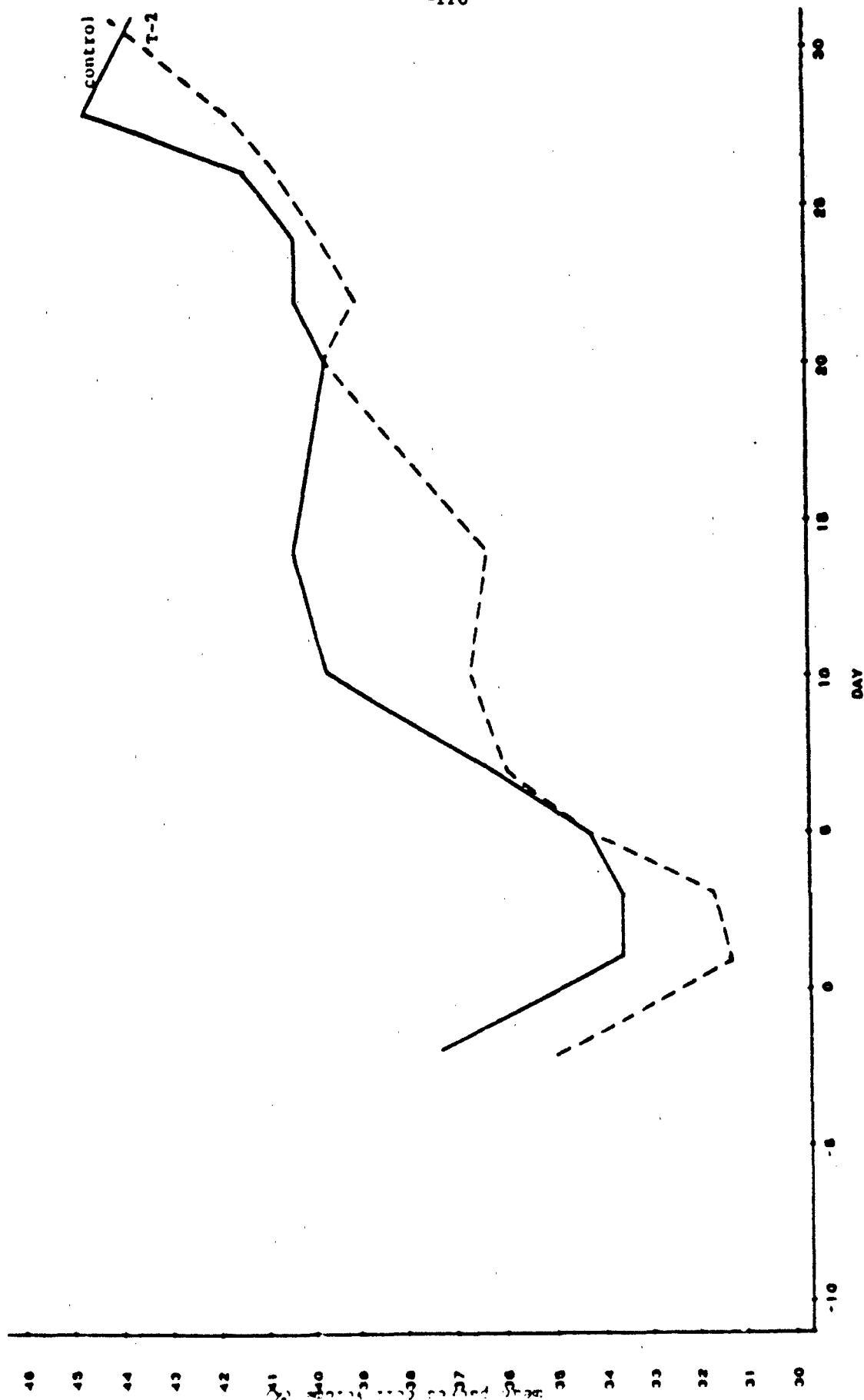
Fig. 11A23  Mean packed cell volume of pigs exposed dermally to T-2 toxin
(15 mg/kg) with vehicle and groups exposed to the vehicle only.
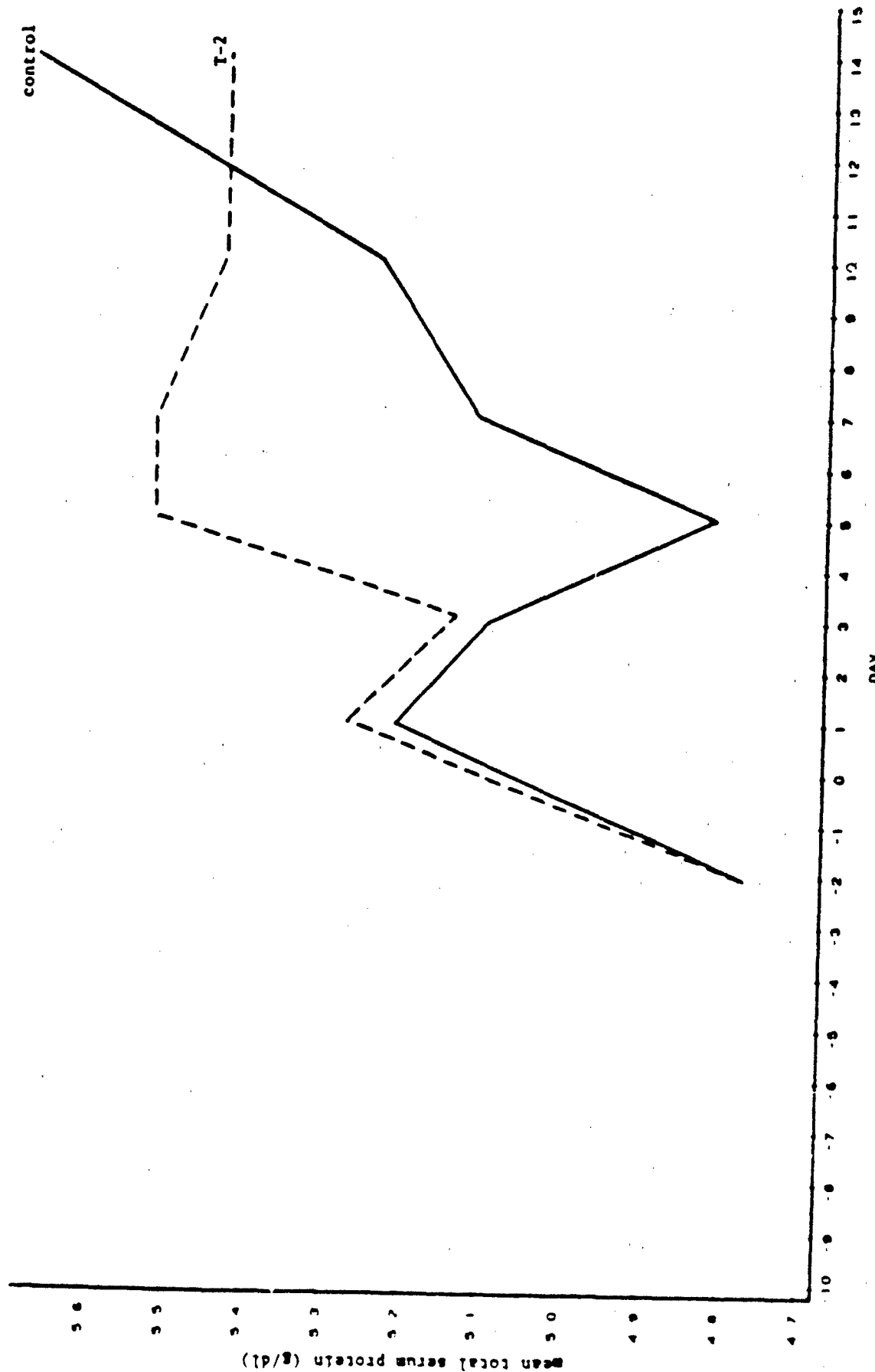
Fig. IIA24  Mean total serum protein of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
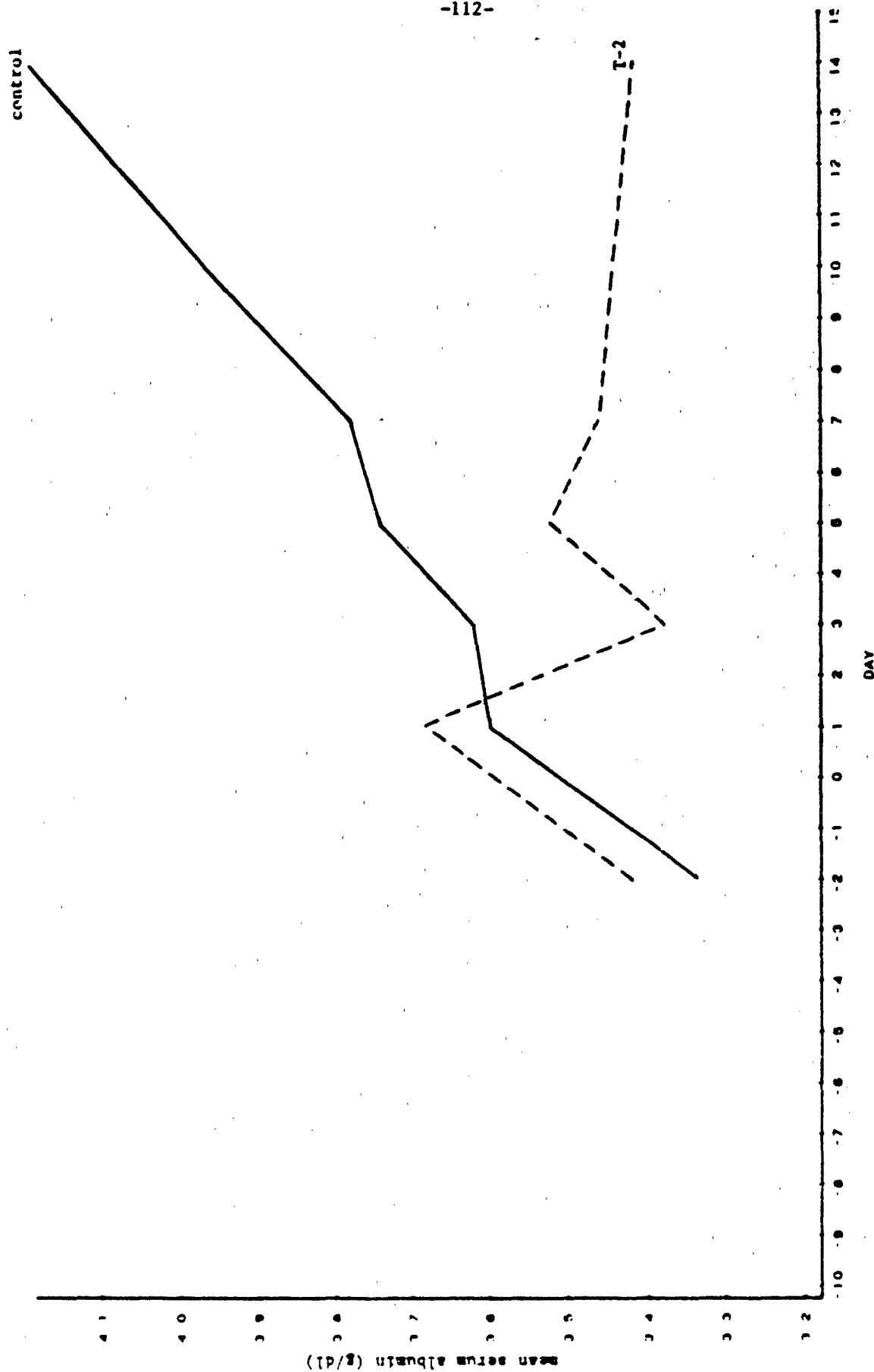
Fig. 1IA25  Mean serum albumin of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
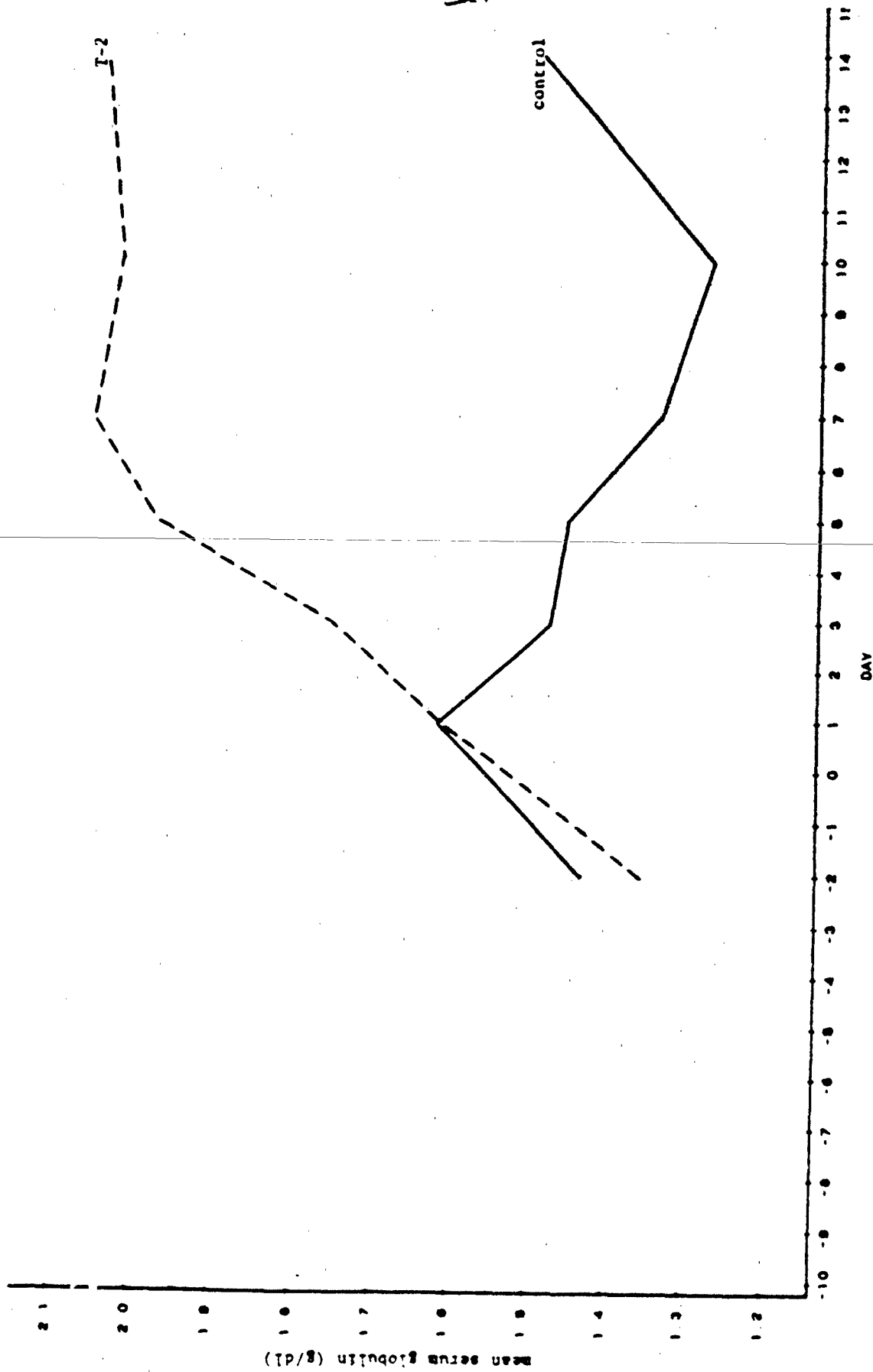
Fig. IIA26  Mean serum globulin of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle groups exposed to the vehicle only.
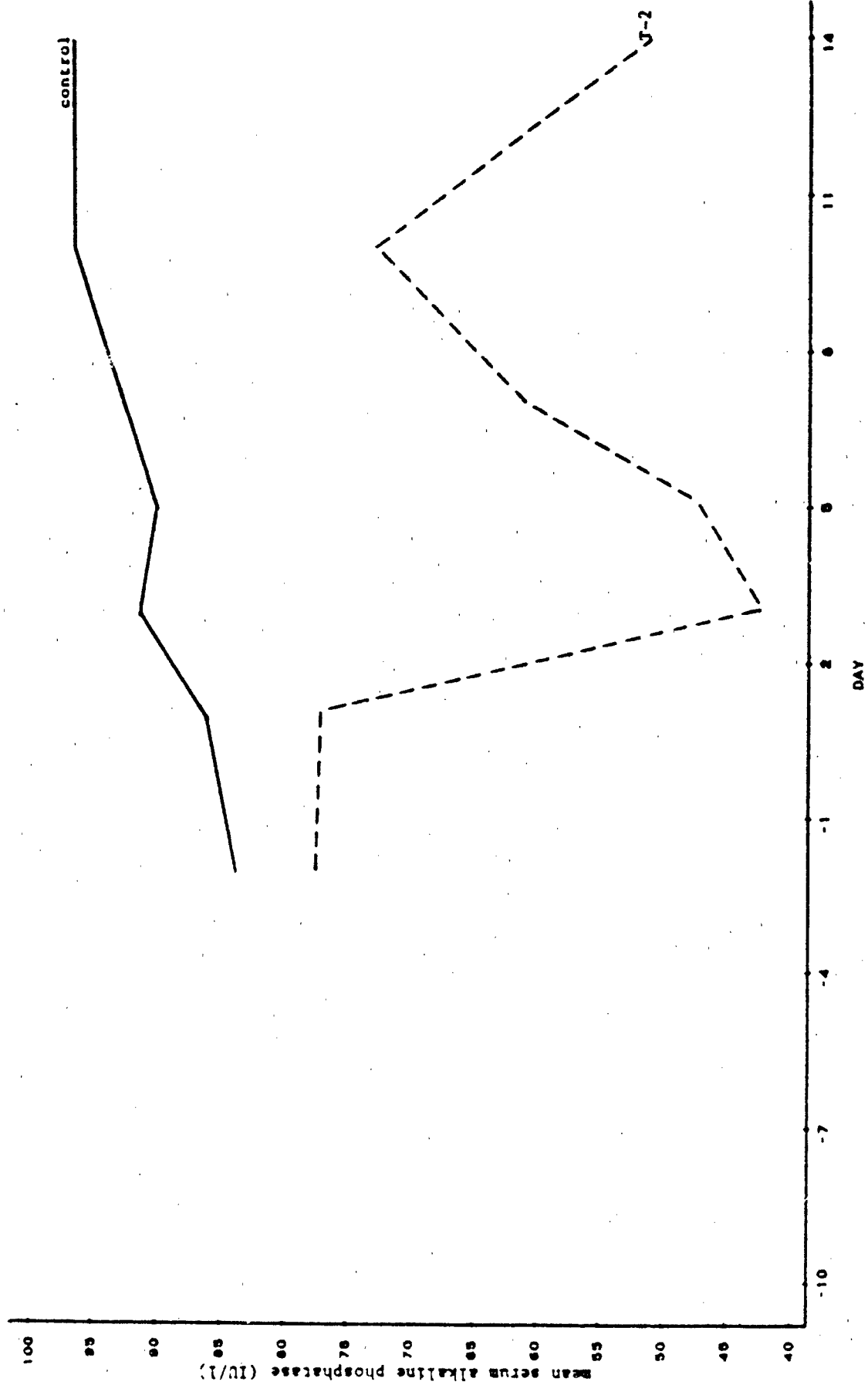
Fig. 11A27  Mean serum alkaline phosphatase of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
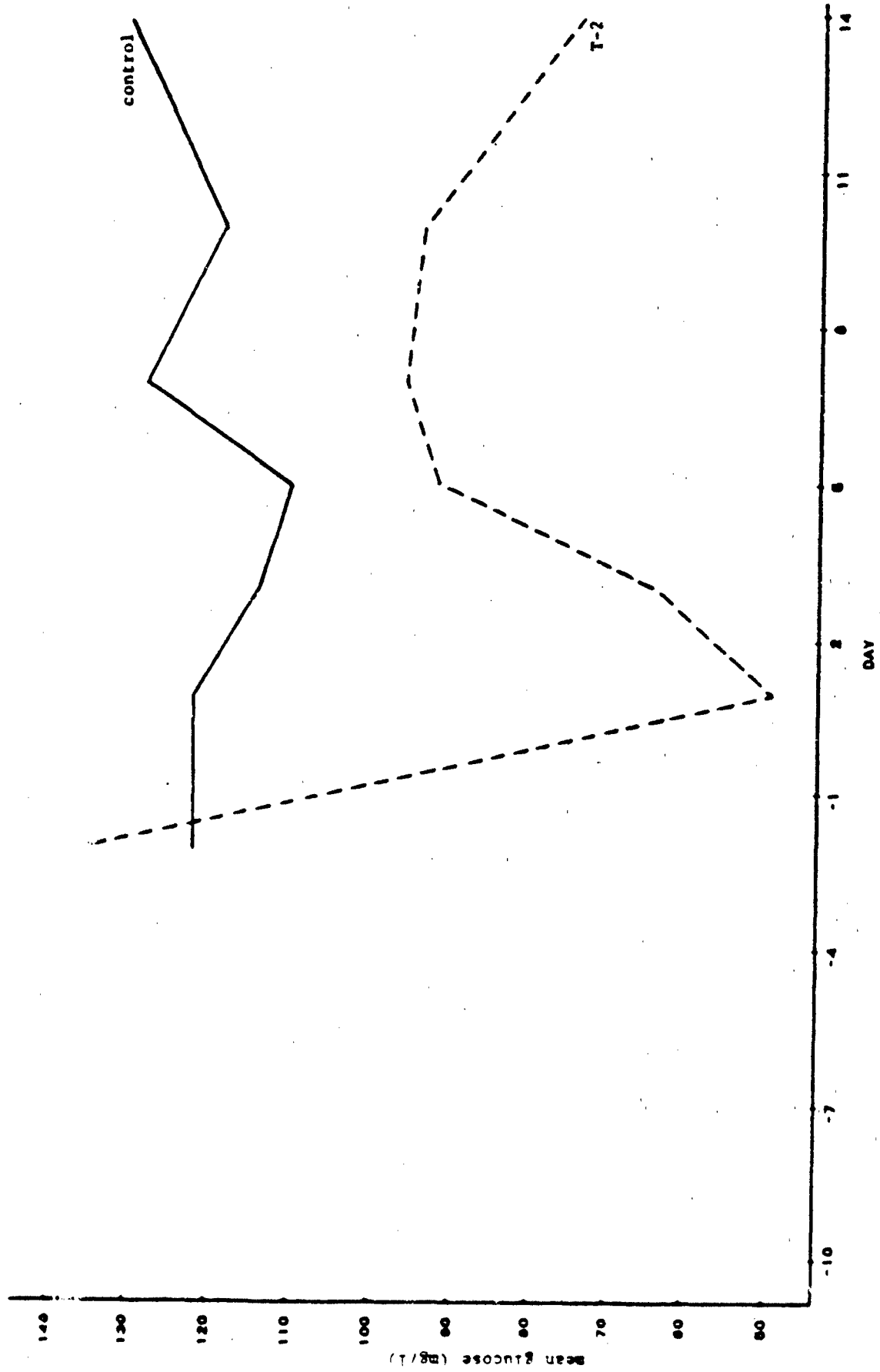
Fig. IIA28  Mean blood glucose of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and groups exposed to the vehicle only.
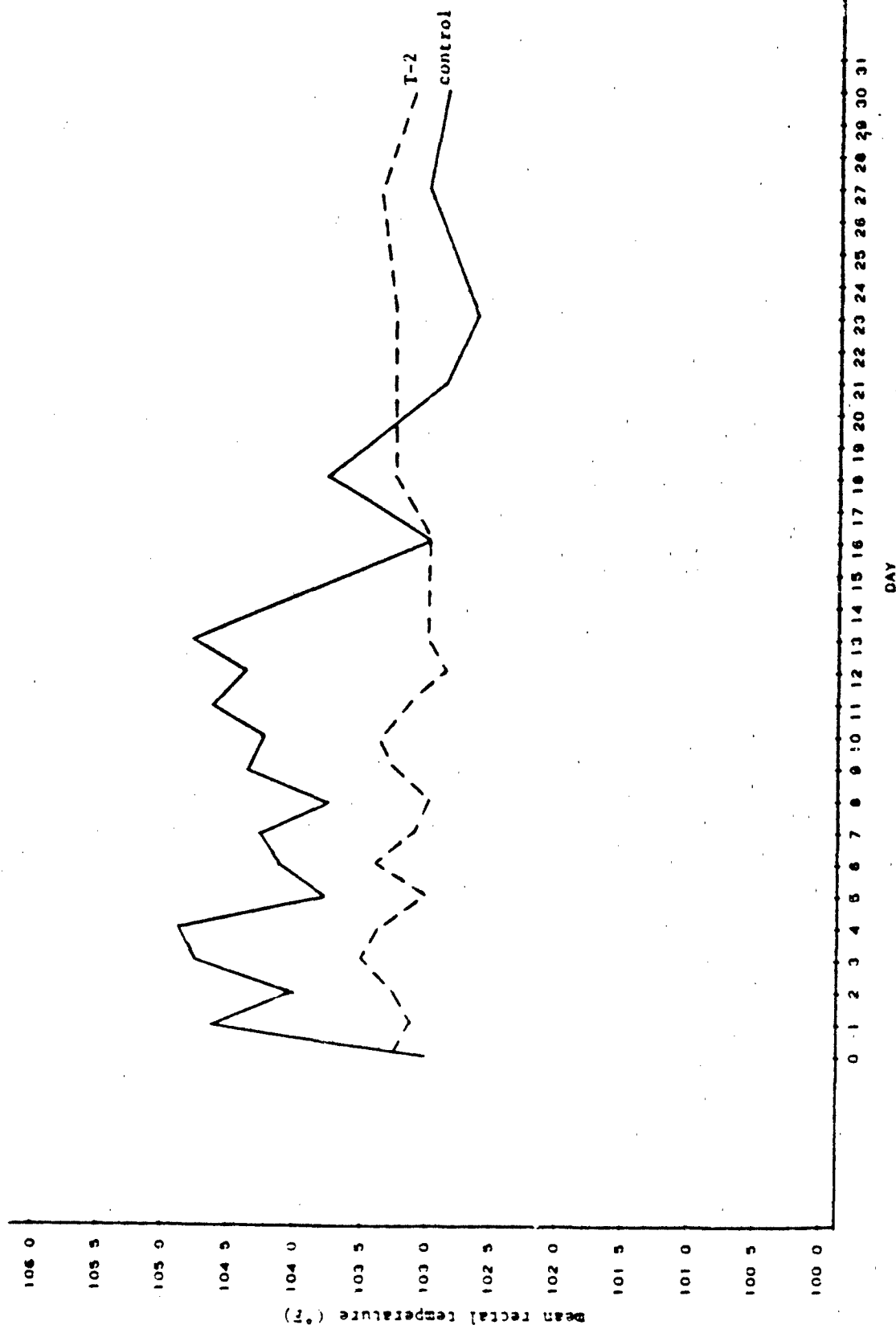
Fig. IIA29  Mean rectal temperature of pigs exposed dermally to T-2 toxin
(15 mg/kg) with vehicle and groups exposed to the vehicle only.
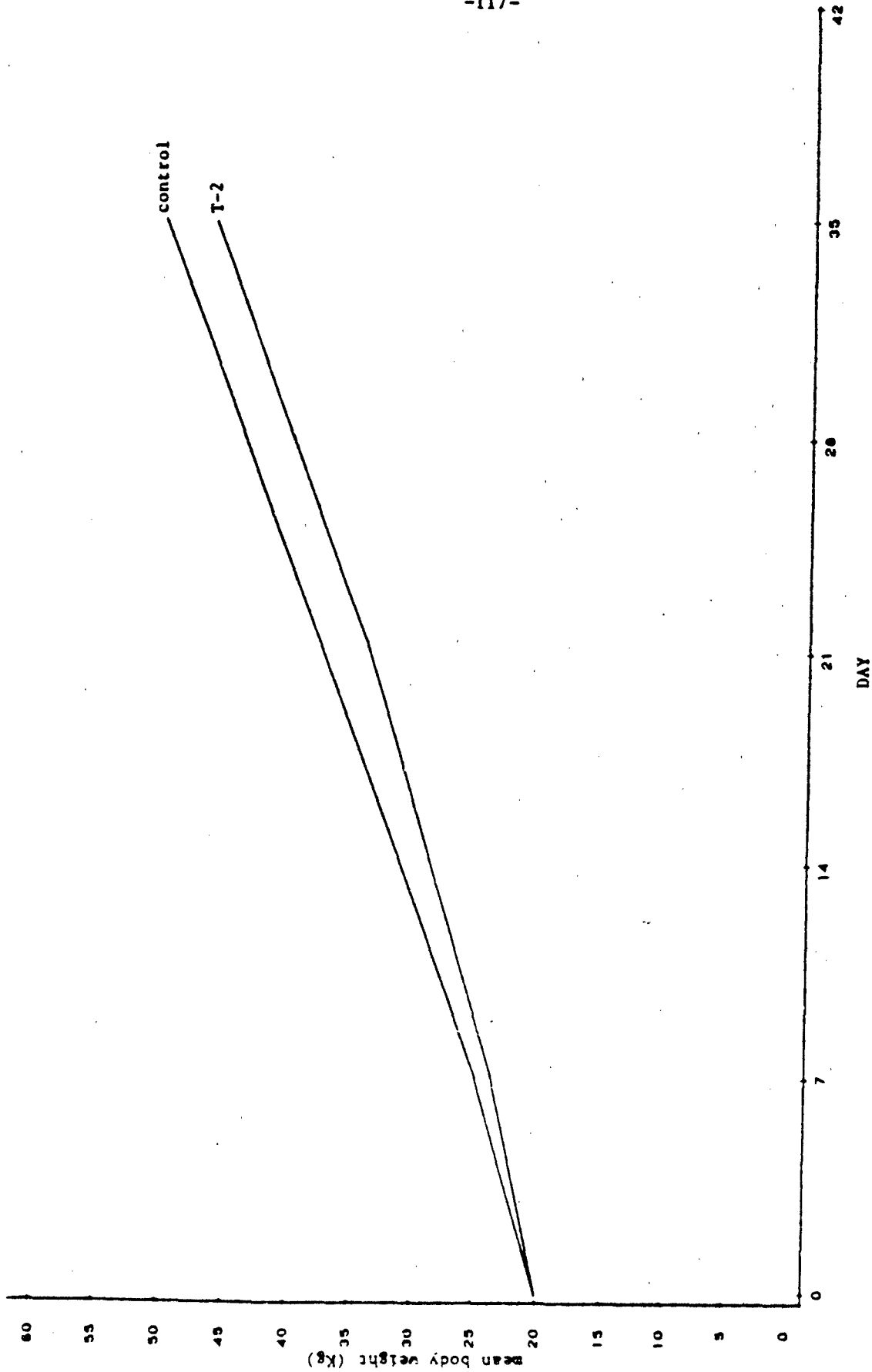
Fig. IIA30  Mean body weight of pigs exposed dermally to T-2 toxin
(15 mg/kg) with vehicle and groups exposed to the vehicle only.

B.   Inhalation Studies

OBJECTIVES

1)  To determine the sequential morphological changes in the lungs and other organs after a single, sublethal dose of T-2 toxin administered by the inhalation route.

2)  To compare the morphological changes induced by the inhalation and dermal routes of exposure.

3)  To determine the possible effects of inhaled T-2 toxin on both local and systemic immunity.

4)  To compare the effects of inhaled and topically applied T-2 toxin on the immune system.

PRELIMINARY STUDY

One preliminary study was carried out in order to set up the techniques of intrabronchial immunization, and to determine whether intrabronchial immunization could induce a systemic immune response. Two pigs were given two doses of SRBC, 21 days apart via the intrabronchial route. Intrabronchial immunization required anesthesia of the pig and instillation of the SRBC via a fiberoptic gastroscope. The responses to this immunization were measured by lymphocyte transformation assays and hemagglutination tests. Adequate immunization was achieved by this route.

STUDIES REMAINING

A group of 5 SPF pigs will be used to set up and refine techniques of lung lavage and separation of cellular components (including macrophages and lymphocytes) and supernatant fluid, to establish procedural protocols for the pulmonary macrophage function test (phagocytosis) and to determine the base-line of local and systemic immunity of normal pigs. This will be completed in October, 1984.

Formal studies will be started soon after this study and accomplished by March 1985. Based on the results of the dermal study, a tentative experimental design has been developed as follows:

IMMUNIZATION AND SACRIFICE TIME (DAYS)

| | 0* | 1 | 3 | 7 | 10 | 14 | 21** | 24 | 28 | 31 |
|---|---|---|---|---|---|---|---|---|---|---|
| Test Group | 5*** | 5 | 5 | 5 | 5 | | | 5 | 5 | 5 |
| Control Group | 3*** | 3 | 3 | 3 | 3 | | | 3 | 3 | 3 |

* 1st immunization

** 2nd immunization

*** number of pigs

III.   ULTRASTRUCTURAL STUDY OF T-2 TOXICOSIS IN SWINE--Victor F. Pang and
       Wanda M. Haschek-Hock

A.     Histopathologic and Ultrastructural Study of Subacute T-2 Toxin
       Intravenously Dosed in Swine

INTRODUCTION AND OBJECTIVES

Cardiovascular lesions have been observed in field cases of T-2 toxin-associated moldy bean hulls poisoning of horses in Japan (Ueno et al., 1972), in geese experimentally fed a diet containing T-2 toxin for 5 to 10 weeks (Palyusik and Koplik-Kovaces, 1975), and in rats surviving more than a year after several intragastric doses (Schoental et al., 1979). More recently, increased blood pressure and severe cadiovascular abnormalities were reported in a long-term study of rats given 4 doses of T-2 toxin, intragastrically (Wilson et al., 1982). A dose-related decrease of contractability, ECG changes, and ultrastructural damage to myocytes was found in isolated hearts of rats (Yarom et al., 1983). Histologic and ultrastructural changes were reported in hearts of rats given intraperitoneally, a single large or repeated small doses of T-2 toxin (Yarom et al., 1983). In our experience, equivocal myocardial degeneration has also been noted in swine and cattle in a toxicokinetic and toxicodynamic study of T-2 toxin (Beasley, 1983) (Appendix C). Additionally, similar lesions were seen in pigs in our previous cardiovascular studies of acute and subacute T-2 toxicosis. Lesions associated with catheterization in these studies, however, precluded a definitive conclusion.

Pancreatic lesions, aside from the tumors of the endocrine and exocrine pancreas, and the degenerative and reparative lesions of pancreatic ducts, stromal vessels and connective tissue reported in the long term study of T-2 toxin in rats, have never been described in any other species. Exocrine

pancreatic degeneration and necrosis were consistantly seen in pigs in our previous pathophysiology studies of subacute T-2 toxicosis.

The purpose of this study was to elucidate and characterize the histological and ultrastructural changes in the myocardium and pancreas of swine induced by a single, intravenous, sublethal dose (0.6 mg/Kg.) of T-2 toxin.

FORMAL STUDY

Eight, 17 to 18 week old, SPF, crossbred gilts, weighing between 54 and 66 kg, were administered an intravenous dose of 0 (2 pigs) or 0.6 mg/kg (6 pigs) T-2 toxin via the ear vein. They were killed 24 or 48 hours after dosing. Sections of heart and pancreas were taken for both light and electron microscopy.

Upon gross examination, one of the pigs killed at 48 hours had scattered, small, subendocardial hemorrhages in the left ventricle, mainly in the papillary muscles. In addition, there were multiple pinpoint white foci, randomly scattered throughout the myocardium of the entire heart, but most prominently in the left ventricle. In this same pig, severe subcapsular and interlobular edema were diffusely present in the pancreas.

Histologically, the principle myocardial lesions consisted of areas of degeneration and necrosis, along with multifocal interstitial edema, with or without mononuclear cell infiltration (Fig. IIIA1). The changes were noted in all dosed pigs, although individual variations were present (Table IIIA1). Morphologically, there were two distinct types of degeneration which usually did not occur concomitantly in the same myofiber. In the first type, which were more widely distributed, the affected myofibers became strongly eosinophilic or completely hyalinized and their nuclei became pyknotic (Fig. IIIA1). The normal longitudinal striations were replaced by multiple, highly

eosinophilic, and transversely oriented contraction bands (Fig. IIIA2). Granulation, fragmentation, mineralization and segmental to extensive losses of myofibers were apparent in some areas (Fig. IIIA3, 4). Interstitial edema and mononuclear cell infiltration were usually associated with this type of alteration. Although the changes were randomly scattered throughout the heart, it appeared that they were more predominent in the atria, papillary muscles of the left ventricle, and lower left and upper right ventricles (Table IIIA1), particularly in the subendocardial region. There was no correlation between the severity of lesions and the length of survival, but the intensity of mineralization and loss of myofibers were more evident in the pig killed at 48 hours which had visible heart lesions.

TABLE III[A] . Summary of the Severity and Frequency of Histopathologic Changes in the Heart, Pancreas, Thymus, and Liver of Subacute T-2 Toxin Intravenously Dosed Swine

| Time of Survival | Pig No. | F/S | Type of Lesion | Rt Atr | Lt Atr | Upper Lt Ven | Mid Lt Ven | Lower Lt Ven | PAP M | Upper Int Wall | Lower Int Wall | Upper Rt Ven | Lower Rt Ven | Pancreas (Deg/Nec) | | Thymus (Lymphoid Necrosis) | | Liver (Mitotic figures) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 Hours | 1 | F | Deg/ | +++ | +++ | + | + | ++ | ++ | + | + | + | + | F | + | F | + | F | - |
| | | S | Nec | +++ | +++ | + | + | ++ | ++ | + | + | + | + | S | ++ | S | + | S | - |
| | | F | Vacuol | - | - | + | - | ++ | +\|± | - | - | + | - | | | | | | |
| | | S | Vacuol | - | - | + | - | + | +\|± | - | - | + | - | | | | | | |
| | 2 | F | Deg/ | ++ | + | - | - | + | ++ | - | - | + | + | F | + | F | +++ | F | - |
| | | S | Nec | ++ | + | - | - | ++ | + | - | - | +\|+\|+\|± | + | S | + | S | ++ | S | - |
| | | F | Vacuol | - | - | ++ | + | ++ | ++ | +\|± | +\|± | + | + | | | | | | |
| | | S | Vacuol | - | - | + | + | + | +++ | +\|± | +\|± | + | + | | | | | | |
| | 3 | F | Deg/ | +++ | - | +\|+\|+\|± | - | - | +\|+\|± | - | + | + | + | F | + | F | + | F | - |
| | | S | Nec | ++ | - | +\|+\|+\|± | - | + | ++ | +\|± | + | + | + | S | + | S | + | S | - |
| | | F | Vacuol | - | +\|± | +\|+\|+\|± | +\|+\|+\|± | + | ++ | + | + | + | + | | | | | | |
| | | S | Vacuol | - | + | +\|+\|+\|± | +\|+\|+\|± | + | ++ | + | + | + | + | | | | | | |
| | 4 | F | Deg/ | +++ | + | +\|± | - | ++ | ++ | +\|± | + | +++ | +\|± | F | ++ | F | +++ | F | +++ |
| | | S | Nec | ++ | + | +\|± | - | + | + | +\|± | + | ++ | +\|± | S | +++ | S | +++ | S | +\|++ |
| | | F | Vacuol | - | +\|± | + | - | +\|± | +\|± | - | + | + | +\|± | | | | | | |
| | | S | Vacuol | - | +\|± | - | - | + | + | - | + | + | + | | | | | | |
| 48 Hours | 5 | F | Deg/ | +\|+ | +\|+\|± | - | +\|+\|± | ++ | ++ | +\|+\|± | +\|+\|+\|± | - | +\|+\|+\|± | F | + | F | +\|± | F | + |
| | | S | Nec | +\|± | +\|+\|± | - | +\|+\|± | + | + | +\|± | +\|+\|+\|± | + | +\|+\|+\|± | S | + | S | +\|± | S | + |
| | | F | Vacuol | - | - | - | - | - | +\|+\|± | - | - | + | + | | | | | | |
| | | S | Vacuol | - | - | - | - | - | +\|+\|± | - | - | + | - | | | | | | |
| | 6 | F | Deg/ | +++ | - | - | - | - | +\|+\|± | +\|± | - | +\|+\|± | +\|± | F | +\|± | F | - | F | - |
| | | S | Nec | ++ | - | - | - | + | +\|+\|± | +\|± | - | +\|+\|± | - | S | +\|± | S | - | S | - |
| | | F | Vacuol | - | - | - | - | - | - | - | - | - | - | | | | | | |
| | | S | Vacuol | - | - | - | - | - | - | - | - | - | - | | | | | | |

F/S = Frequency/Severity; Rt = Right; Lt = Left; Atr = Atrium; Ven = Ventricle; Pap = Papillary; M = Muscle; Int = Interventricular; Deg = Degeneration; Nec = Necrosis; Vacuol = Vacuolization;

Frequency:  + = Scattered;  + = Multifocal;  ++ = Frequent;  +++ = Very Frequent

Severity:  ± = Minimal;  + = Mild;  ++ = Moderate;  +++ = Severe

WBB:9522D
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The second type of degeneration was vacuolization of myofibers (Fig. IIIA5). The affected myofibers had either a single large, or multiple variable-sized vacuoles. The nuclei were pushed either to one pole or to the lateral side of the myofibers. The vacuoles were clear, or contained a pale-stained, finely granular to fibrillar substance, or occasionally contained well-defined large hyaline droplets (Fig. IIIA6). This ?-sion appeared to have a predilection for the papillary muscles and the free wall of the left ventricle (Table IIIA1). As with the first type of degeneration, no correlation between the severity of vacuolization and length of survival was found.

Ultrastructural changes in the slightly injured myofibers, which did not histologically show transverse contraction bands, vacuolization, or necrosis, consisted of areas of intermyofibrillar edema with dispersion of myofibrils, loss of Z and M bands, and glycogen accumulation (Fig. IIIA7). In the areas showing transverse contraction bands with light microscopy, there were numerous, dense, necrotic myocytes. These myocytes had clumps of eletron-dense and disrupted contractile material which was usually surrounded by large numbers of mitochondia (Fig. IIIA8, 9). Lysis of filaments, edema, and accumulation of finely granular and fibrillar substances, were noted in between these dense clumps of disrupted contractile material (Fig. IIIA10). Focal distension of the elements of sarcoplasmic reticulum was seen in some affected myocytes (Fig. IIIA8). The nuclei of necrotic myocytes showed prominent invagination and irregularity of the nuclear membrane with clumping and margination of heterochromatin (Fig. IIIA). The vacuolized myofibers observed histologically, had diffuse, variable distension of the elements of sarcoplasmic reticulum (Fig. IIIA11, 12). These vacuoles contained varying amounts of a finely granular, fibrillar substance. In extremely dilated

vacuoles, aggregates of membranous fragments were often present at the margins of the vacuolar spaces (Fig. IIIA12). Connections appeared to be present between the membranous aggregates and the membrane lining the space. This may suggest that the extremely distended vacuoles were formed by coalescence of the multiple, adjacent affected elements of sarcoplasmic reticulum. In these injured myocytes, lysis and destruction of the myofibrils with accumulation of large quantities of disorganized fibrillar substance were evident, but no hypercontraction bands were observed (Fig. IIIA13). Mitochondria of these myocytes contained variable numbers of amorphous electron-dense granules (Fig. IIIA13).

Pancreatic changes consisted of multifocal degeneration and necrosis of single, or grouped acinar cells in all pigs killed at 24 hours (Fig. IIIA14). The affected cells were vacuolized by single large vacuoles or, occas..onally, multiple variable-sized vacuoles. The nuclei were pushed eccentrically and became pyknotic. Globular condensation of a portion of the cytoplasm (separated from the remainder by a halo) was seen in many of the degenerating cells. The condensed globular cytoplasm often became hyalinized. Nuclear pyknosis and karyorrhexis were apparent in some of the injured cells. Areas of interlobular edema and fibrin deposition, along with fat necrosis, were observed in two pigs of this group. The islets of Langerhans were relatively unaffected, although occasional single cell degeneration, as vacuolization and increased cytoplasmic granularity, was observed.

Similar pancreatic changes were observed in two treated pigs killed at 48 hours, but the changes were very severe and a diffuse, suppurative, necrotizing, pancreatitis was present in one pig, which had grossly evident pancreatic edema (Fig. IIIA15). The lobules were indistinct and the acini were disorganized and disrupted. Edema, fibrin deposition, and infiltration

of neutrophils and macrophages were prominent, along with fat necrosis and scattered small, focal, intralobular hemorrhages. Hyperplasia, characterized by cryptal formation and common mitotic figures was seen in the ductular and ductal epithelia. The number of islets was markedly decreased. The remaining islets were relatively intact, but occasional cellular swelling was present.

The degenerating pancreatic acinar cells showed ultrastructural changes including variable distension of the rough endoplasmic reticulum and perinuclear cistern, which was eventually disrupted and coalesced to form large membrane-bound spaces (Fig. IIIA16, 17). Small, irregular, membrane-lining vacuoles, myelin figure-like membrane aggregates, and small, membranous fragments were often present in these large membrane-bound spaces. The remaining area of these spaces, were either clear, or contained an electron-lucent granular substance. The mitochondria were swollen, lost cristae, and finally became disrupted. The zymogen granules were irregular, smudgy, and electron-lucent. In necrotic cells, the nuclear remnants were pushed eccentrically by large cytoplasmic vacuoles and were surrounded by a disintegrated nuclear envelope with a markedly dilated perinuclear cistern.

DISCUSSION

Based on histologic and ultrastructural findings, the myocardial lesions of T-2 toxicosis in swine were characterized by two types of myocytic alterations: 1) early myofiber degeneration, characterized by sarcoplasmic edema, malaligned myofibrils, and loss of Z and M bands, and accumulation of glycogen; followed by myofiber necrosis, with hypercontraction bands, myofibrillar lysis and nuclear damage, and subsequent mineralization and loss of myofibers, 2) sarcoplasmic vacuolization, characterized by distension of the sarcoplasmic reticulum, with myofibrillar lysis and amorphous electron-dense mitochondrial deposition.

Necrosis with contraction bands has been described in many forms of cardiac injury in which excessive amounts of calcium from the blood enter into myocytes and trigger a number of events, including hypercontraction, decreased mitochondrial energy production, formation of intramitochondrial calcium deposits, and activation of sarcoplasmic phospholipase and proteases (Dayton and Schollmeyer, 1980; Toyo-Oka and Masaki, 1979). Among these injuries, the lesions induced by catecholamines have been well described (Ferrans et al., 1969; Ferrans et al., 1970; Ferrans, 1972; Rona et al., 1977). Isoprotererol (ISO), the synthetic catecholamine, has been demonstrated to increase the phospholipase and phosphoprotein lipase activities in isolated sarcolemmae of heart muscle cells (Franson et al., 1978; Franson et al., 1977) and to stimulate sarcolemmal lipolytic activity (Franson et al., 1976; Balazs, 1973). The enhanced lipolytic activity, in turn, results in increased sarcolemmal permeability that permits toxic calcium influx (Weglicki et al., 1972).

There is a strong tendency for localization of the ISO-induced myocardial lesion in the endocardial third or half of the myocardium (Godfraind and Sturbois, 1979). The tendency is more pronounced in (Godfraind and Sturbois, 1979; Balazs et al., 1971; Van Vliet et al., 1966), or is limited to (Van Vliet et al., 1977), the left ventricle, although lesions are also present in the right ventricle. It is well known that perfusion pressure is lowest in the subendocardium, because coronary pressure decreases, while tissue pressure increases, as one moves from the epicardium toward the endocardium (Moir, 1972). The toxic effects of catecholamines include positive chronotropic and negative inotropic actions, as well as hypotension (Balazs and Bloom, 1982). Tachycardia decreases the period of perfusion, whereas hypotension may lower coronary perfusion. Positive inotropic effects result in greater expenditure of energy and, therefore, greater requirements for ATP production. All of

these effects, then, lead to a condition of relative coronary insufficiency, which is one hypothetical mechanism by which the focal necrosis occurs (Balazs and Bloom, 1982). It is also suggested that cellular edema resulting from prior cellular injury by other mechanisms results in capillary compression and subsequent non-flow. Thereafter, the severe reduction in microvascular flow aggravates the existing injury, and may even be responsible for additional injury of a purely ischemic nature (Balazs and Bloom, 1982).

T-2 toxin has been shown to cause changes consisting of decreased contractility and electocardiographic abnormalities without much effect on heart rate, or QRS and QT intervals (Yarm et al., 1983). Ultrastructurally, the affected myofibers and their mitochondria were swollen. The basement membrane of injured myocytes was intact, while the sarcolemma was often distorted, vacuolized, or absent. Dramatically increased plasma concentrations of catecholamines, including both epinephrine and norepinephrine, were seen in our previous study of cardiovascular functional alterations of intravascularly lethal (4.8 mg/kg) and sublethal (0.6 mg/kg) doses of T-2 toxin in swine. Large areas of cellular infiltration and patchy fibrosis, mainly in the subendocardial region of the left ventricle, were described in rats killed 1 or 2 months after a series of small doses of T-2 toxin (Yarm et al., 1983). In the present study, myocardial degeneration, necrosis, and mineralization, also were present, mainly in the subendocardial region, although aside from the left ventricle, lesions were observed in the right venticle and both atria as well. The changes of affected myocytes were compatable with those induced by catecholamines.

Profound cytoplasmic vacuolization, mainly due to pronounced swelling of the tubules and cisterns of the sarcoplasmic reticulum, is one of the two forms of degeneration occurring n the cardiac myocytes in chronic

anthracycline toxicosis (Ferrans, 1978). Extensive sarcoplasmic vacuolization has also been observed in the atrial myocardium of pigs with acute monensin toxicosis (Van Vliet and Ferrans, 1984). Peroxidative damage of membrane was cited as one of the possible mechanisms in both instances. Although T-2 toxin is not known to exert direct peroxidative effects, accelerated phospholipid degradation and lipid peroxidation of plasma membranes have been suggested as one mode of action of T-2 toxin (Yarm et al., 1983). In the present study, the papillary muscles of the left ventricle were the areas with the most severe myocytic vacuolization. The oxygen demand of the left ventricular muscles is high because of their great mechanical work during systole (Balazs and Bloom, 1982). They are supplied by end-arteries and since they are thickest in the subendocardial region, the capillary supply per unit mass is lower than elsewhere in the subendocardium. Because the capillary bed in the papillary muscles is near maximal utilization, even at rest, hypoxia develops when demand increases. This hypoxia is probably more severe, and of longer duration than that of other subendocardial tissues (Debusk and Harrison, 1969; Roberts and Cohen, 1972). In T-2 treated pigs, it is very possible that anatomic and functional specificities of the papillary muscles of the left ventricle predispose these tissues to membranous damage with resultant development of distension of the sarcoplasmic reticulum.

T-2 toxin given as a single, intravascular dose to swine produced a shock syndrome in our previous study. It was characterized by a decline in cardiac output and blood pressure, increased plasma concentrations of epinephrine, norepinephrine, thromboxane B , 6-keto-$PGF1alpha$ and lactate, as well as decreased arterial pH and arterial oxygen partial pressures (Lorenzana et al., 1984) (Appendix A & B). The hypotension associated with shock usually results in hypoperfusion of the splanchnic region, especially the pancreas (Lefer and

Curtis, 1982). Pancreatic blood flow decreases abruptly in hemorrhagic shock (Forsythe et al., 1970; Lefer and Spath, 1974; Spath et al., 1974). The pancreatic vasculature appears to be sensitive to alterations in cardiovascular status and uniformly becomes ischemic in shock (Lefer and Curtis, 1982). It has been suggested that defects in cell membranes, presumably due to activation of endogenous phospholipases and/or inhibition of the reacylation of lysophospholipids, are an early feature of irreversible ischemic cell injury (Ashraf and Halverson, 1977; Beller et al., 1976; Chien et al., 1979; Coleman et al., 1976; Farber et al., 1978; Farber and Young, 1981; Flores et al., 1972; Flores et al., 1972; Leaf, 1973; MacKnight and Leaf, 1977; Farber, 1982). The plasma membranes and the membranes of the endoplasmic reticulum in liver cells and the sarcoplasmic reticulum in myocardial cells are preferentially affected (Chien et al., 1979; Farber, 1978; Farber and Young, 1981; Chien et al., 1978; Chien and Farber, 1977; Chien et al., 1981).

Distension of the endoplasmic reticulum was the most evident morphological alteration of the pancreatic acinar cells occurring in our T-2 dosed pigs. It is speculated that ischemia, secondary to hypoperfusion, might play an important role in causing damage to the pancreatic acinar cells of T-2 dosed animals. In addition, hypoxia and ischemia are potent stimuli for disruption of lysosomal membranes and for activation of zymogenic proteases (Lefer and Curtis, 1982). The result is the release of large amounts of proteolytic enzymes into the cytoplasm of pancreatic acinar cells. Acidosis can enhance the activity of the lysosomal proteases once they are released from lysosomes (Glenn and Lefer, 1971). The zymogenic and lysosomal proteases released during the shock process appear to facilitate proteolysis and thereby stimulate myocardial depressant factor (MDF) production (Lefer and Barenholz,

1972; Litvin et al., 1973). MDF is released from acinar cells through damaged cell membranes. The peptide may bind to large carrier proteins in the extracellular fluid, or it may remain as a free peptide which is taken up by lymphatic capillaries, or is transported directly by capillaries to the systemic circulation (Glenn and Lefer, 1970; Lefer, 1978). The best known action of MDF is its negative inotropic effect in both isolated papillary muscles (Lefer, 1974) and in the hearts of intact animals (Glenn and Lefer, 1971; Wangensteen, 1970). The pig which had severe, diffuse, necrotizing pancreatitis was the only one showing gross myocardial lesions. MDF released from damaged pancreatic acinar cells might have served to augment the cardiac dysfunction in T-2 treated pigs.

Fig. IIIA1 Heart of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. Focal myocardial degeneration and necrosis is characterized by strong eosinophilia, hyalinization, contraction bands and nuclear pyknosis along with interstitial edema and minimal mononuclear cell infiltration. HE stain, 690 x.

Fig. IIIA2 Heart of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. There is focal myocardial degeneration and necrosis with prominent contraction bands and moderate interstitial edema. PTAH stain, 750 x.
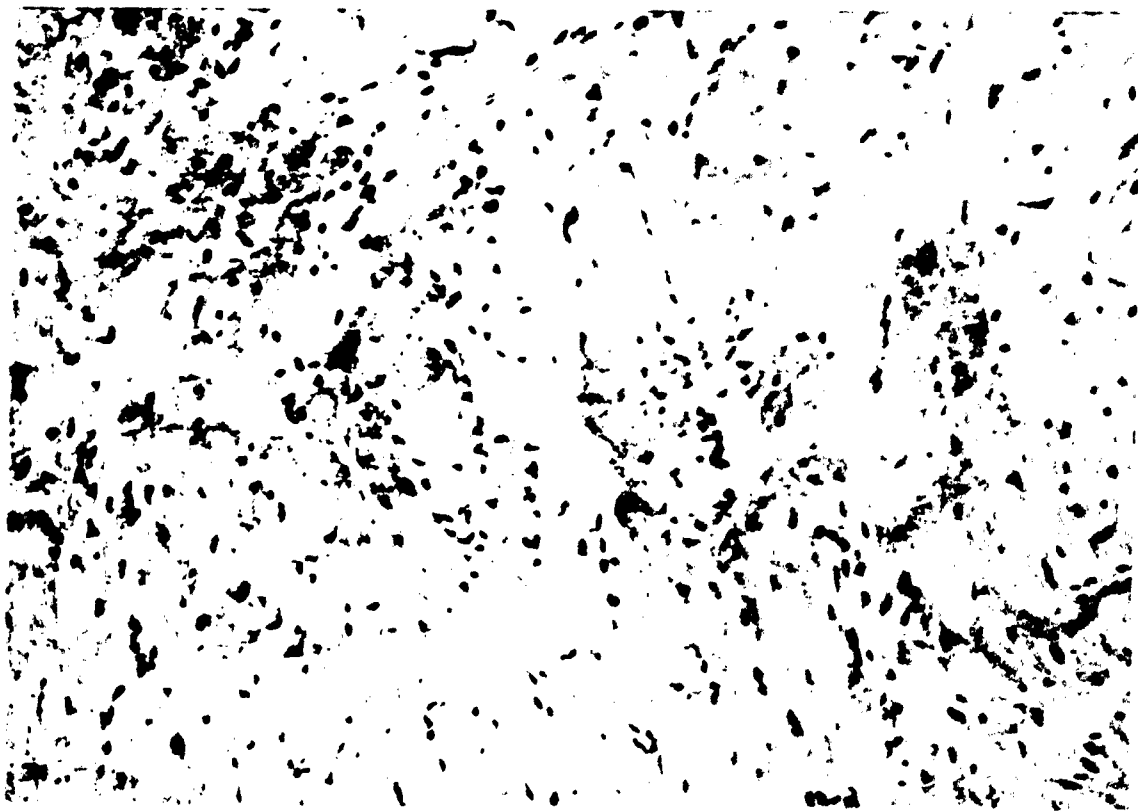
Fig.   IIIA3   Heart  of a pig given T-2 toxin (0.6 mg/kg)  intravenously  and
               killed  48  hours later.   There  is focally extensive loss  of
               myofibers.   PTAH stain, 660 x.

Fig.   IIIA4   Heart  of a pig given T-2 toxin (0.6 mg/kg)  intravenously  and
               killed 48 hours later.   There  is  focally extensive myocardial
               necrosis and mineralization.   HE Stain, 750 x.
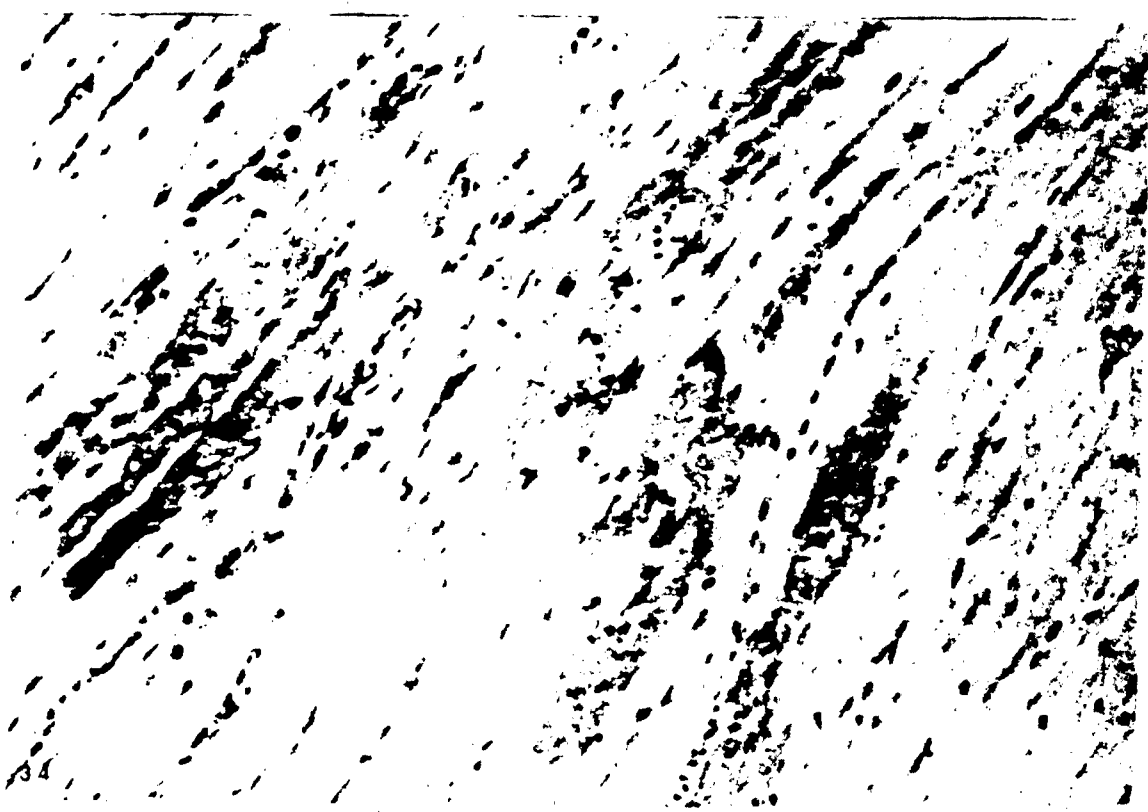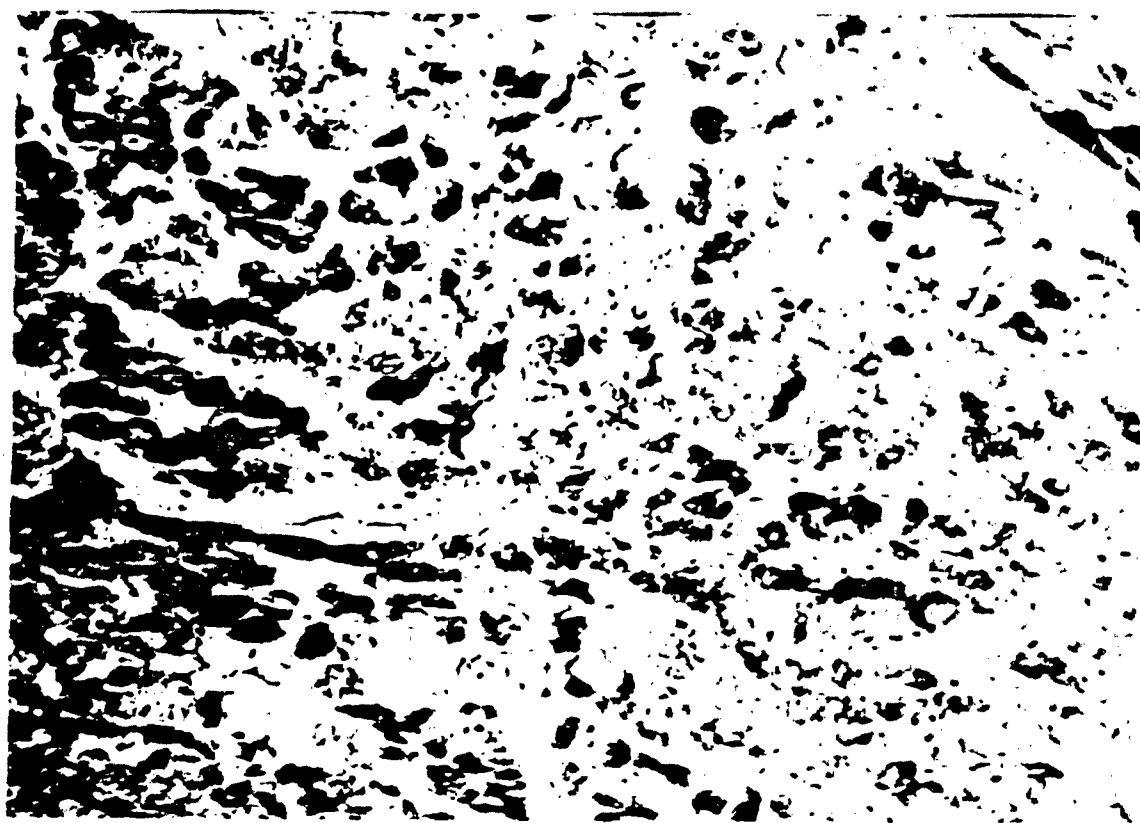
Fig.  IIIA5  Heart of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. There is multifocal myofiber vacuolization. HE stain, 250 x.

Fig.  IIIA6  Heart of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. Myofiber vacuolization is characterized by a single large or multiple variable-sized vacuoles with eccentric nuclei. The vacuoles are either clear or contain finely granular substance. HE Stain, 1500 x.
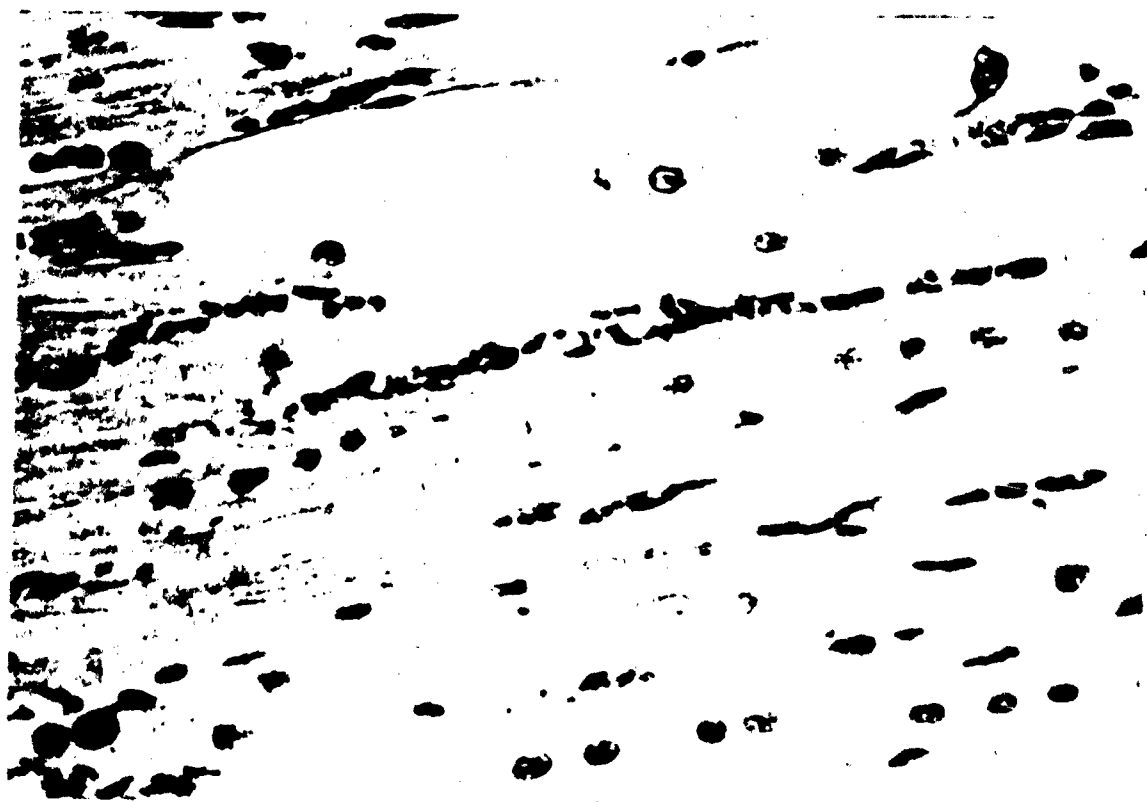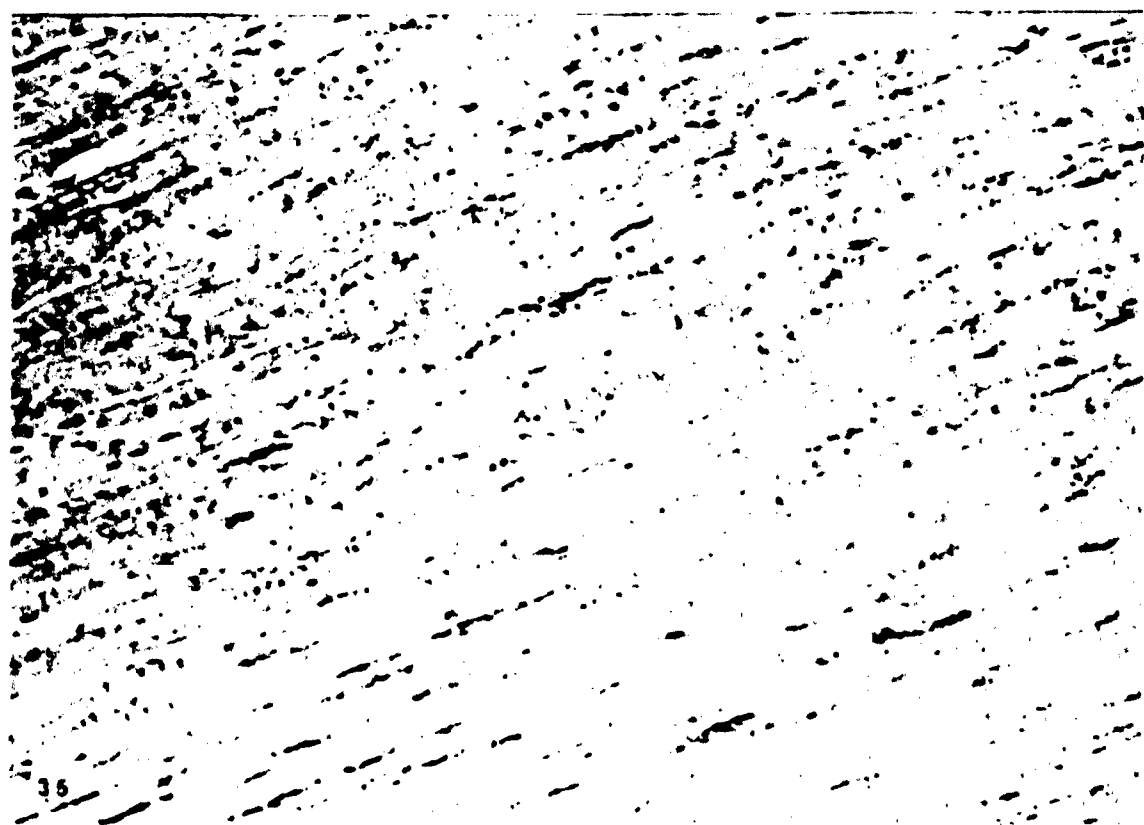
Fig. IIIA7 Papillary muscle of the left ventricle of a pig given T-2 toxin (0.6 mg/kg) and killed 24 hours later. The injured myofiber shows dispersion of myofibrils by intermyofibrillar edema along with loss of Z and M bands as well as glycogen deposition. The myofiber at the right upper corner is relatively normal, 10,400 x.

Fig. IIIA8 Left atrium of a pig given T-2 toxin (0.6 mg/kg) and killed 24 hours later. The myocytes have clumps of electron-dense and disrupted contractable material which are surrounded by large numbers of mitochondria along with focal distension of sarcoplasmic reticulum, 6530 x.
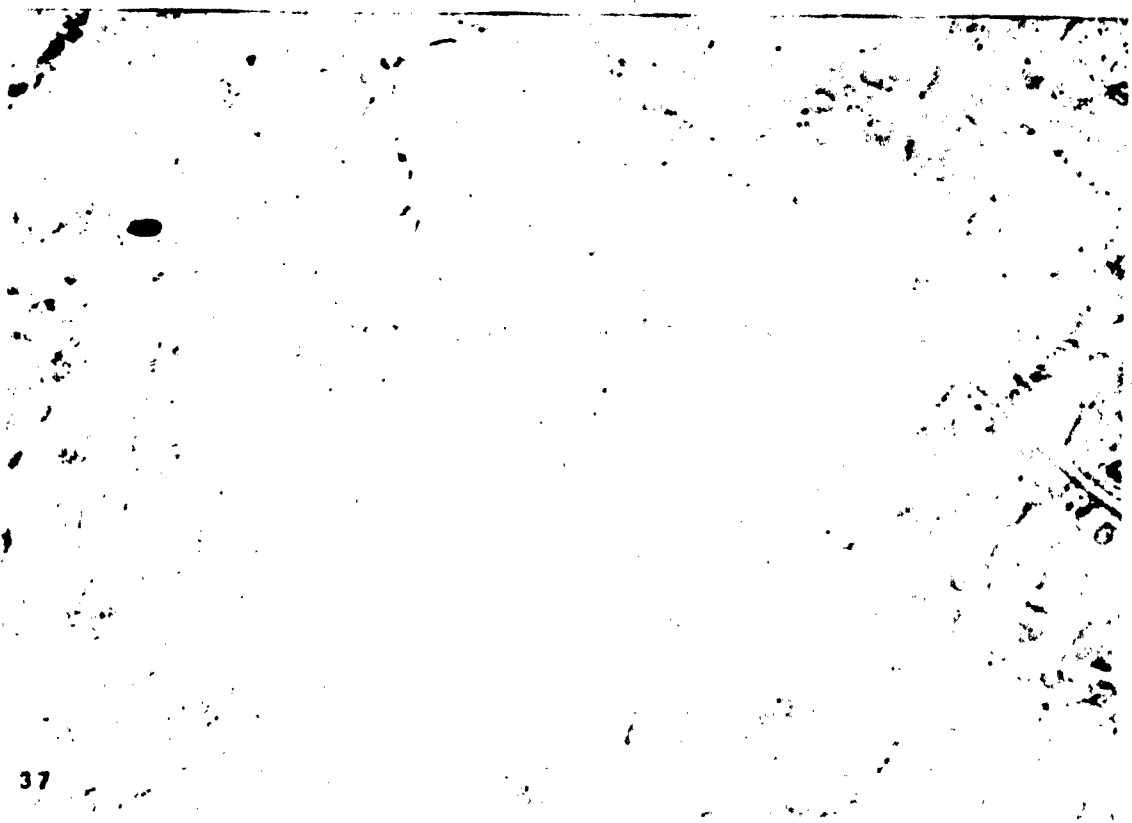
37
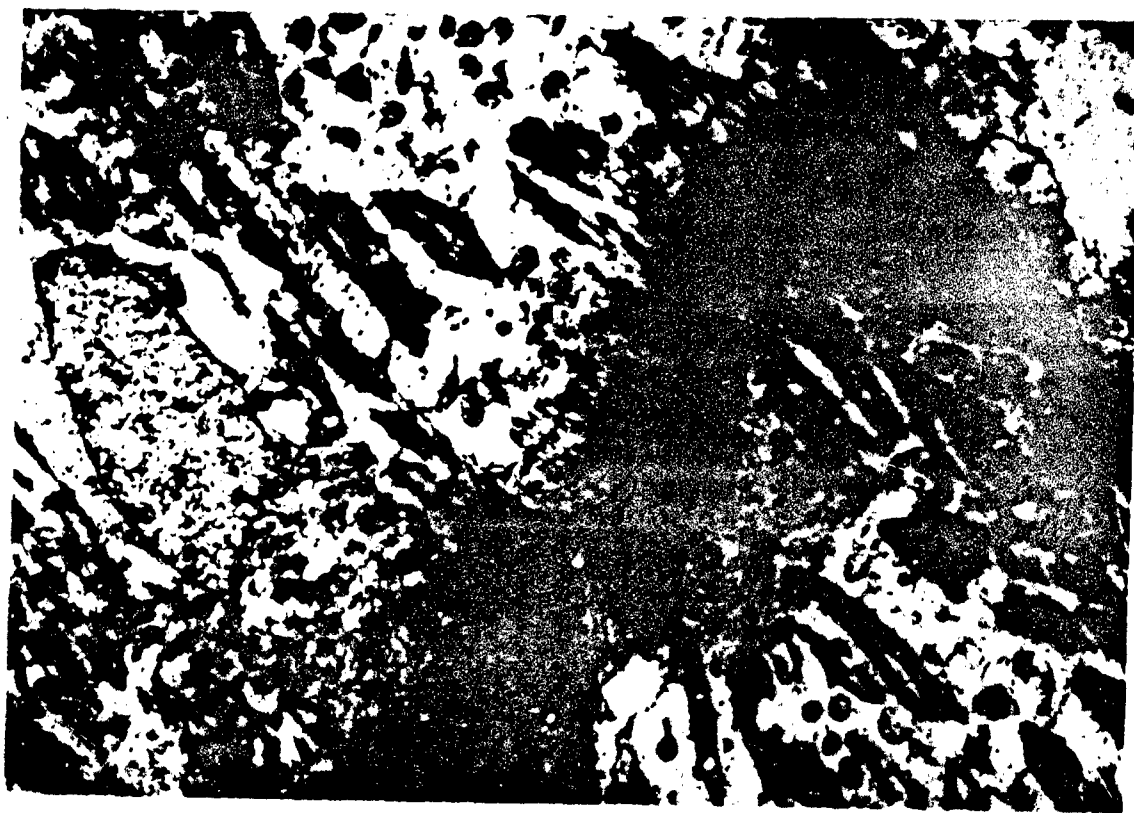
Fig.    IIIA9    Left atrium of a pig given T-2 toxin (0.6 mg/kg) and  killed  24
                hours later.  The myocytes contain clumps of electron-dense  and
                disrupted material which  are  surrounded  by  large  numbers of
                mitochondria along with areas of lysis of filaments. The nucleus
                has  prominent invagination  and  irregularity  of  the nuclear
                membrane with clumping and margination  of  the heterochromatin,
                6530 x.

Fig.    IIIA10   A  higher magnification of Fig.  IIIA9  having  the  disrupted
                electron-dense  contractile  material  with  areas of lysis of
                filaments and accumulation of  finely granular  and  fibrillar
                substance, 13300 x.

Fig. IIIA11 Papillary muscle of the left ventricle of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. The myofibers contain multiple variably distended elements of sarcoplasmic reticulum and have intermyofibrillar edema and myofibrillar lysis, 3600 x.

Fig. IIIA12 Papillary muscle of the left ventricle of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. The myofiber shows myofibrillar lysis, and widely distended sarcoplasmic reticulum with aggregates of membranous fragments present at the margin of the space, 4800 x.
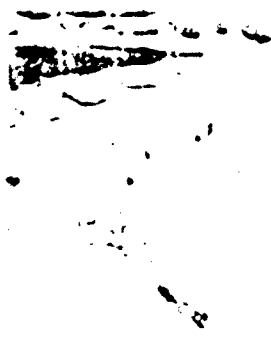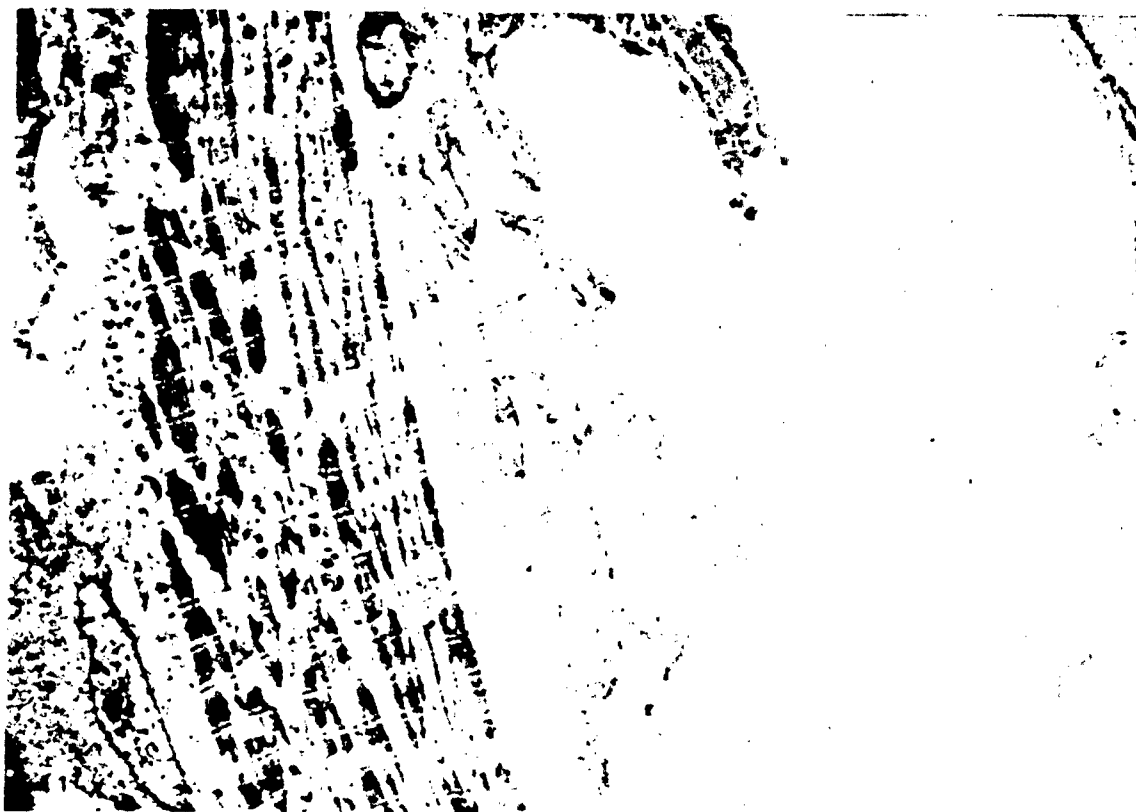
Fig. IIIA13 Papillary muscle of the left ventricle of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. The vacuolized myocyte has lysis and destruction of the myofibrils with accumulation of large quantities of disorganized fibrillar substance. The mitochondria contain amorphous electron-dense granules. No contraction bands present, 34000 x.

Fig. IIIA14 Pancreas of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. There is multifocal vacuolar degeneration and necrosis of single or grouped acinar cells. HE stain, 1450 x.

43

44
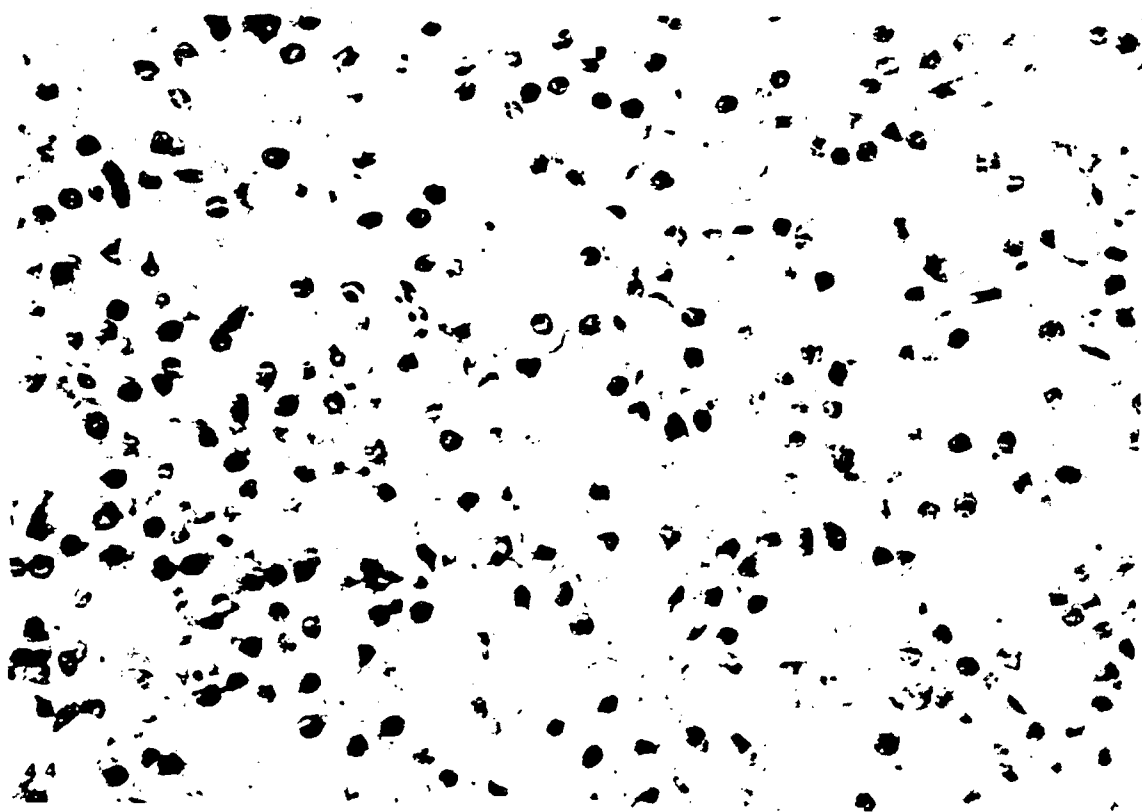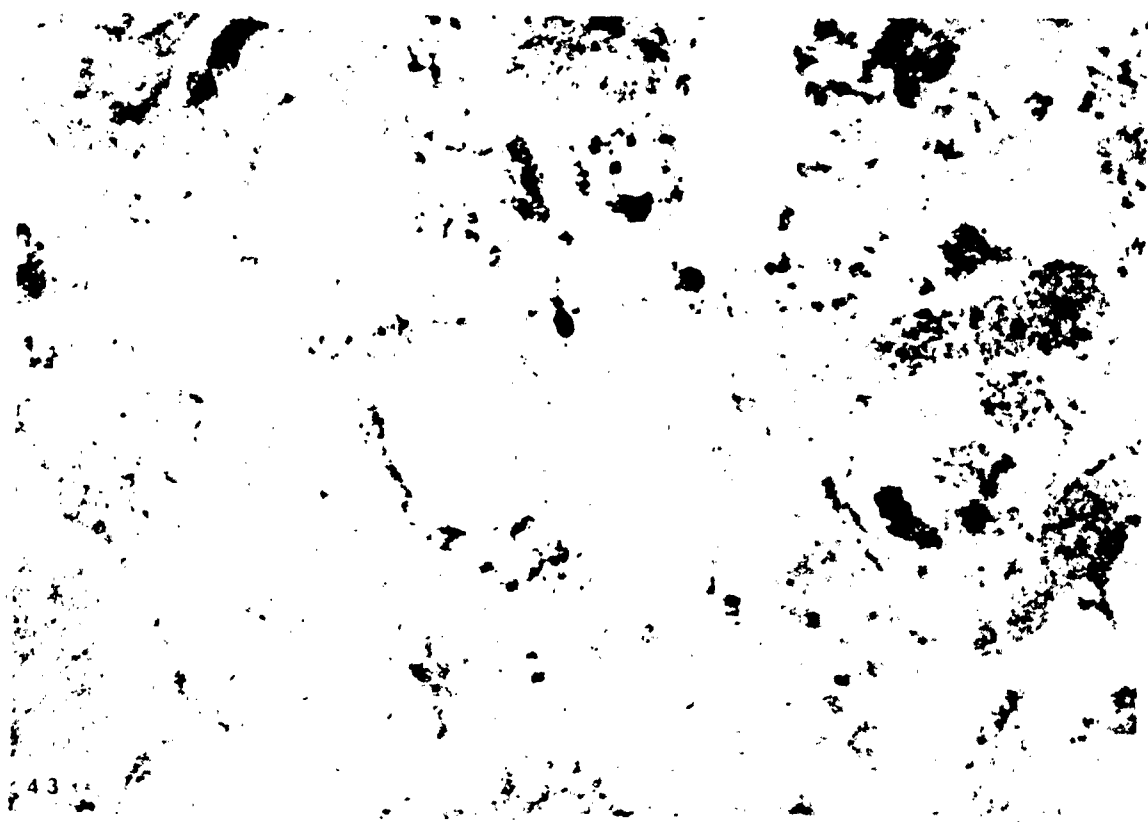
Fig. IIIA15    Pancreas of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 48 hours later. There is diffuse suppurative necrotizing pancreatitis characterized by disorganization and necrosis of acini, intralobular and interlobular edema, fibrin deposition, with infiltration of neutrophils and macrophages, and areas of fat necrosis. HE stain, 580 x.

Fig. IIIA16    Pancreatic acinar cells of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. There is variable distension of the rough endoplasmic reticulum and perinuclear cistern, 26000 x.
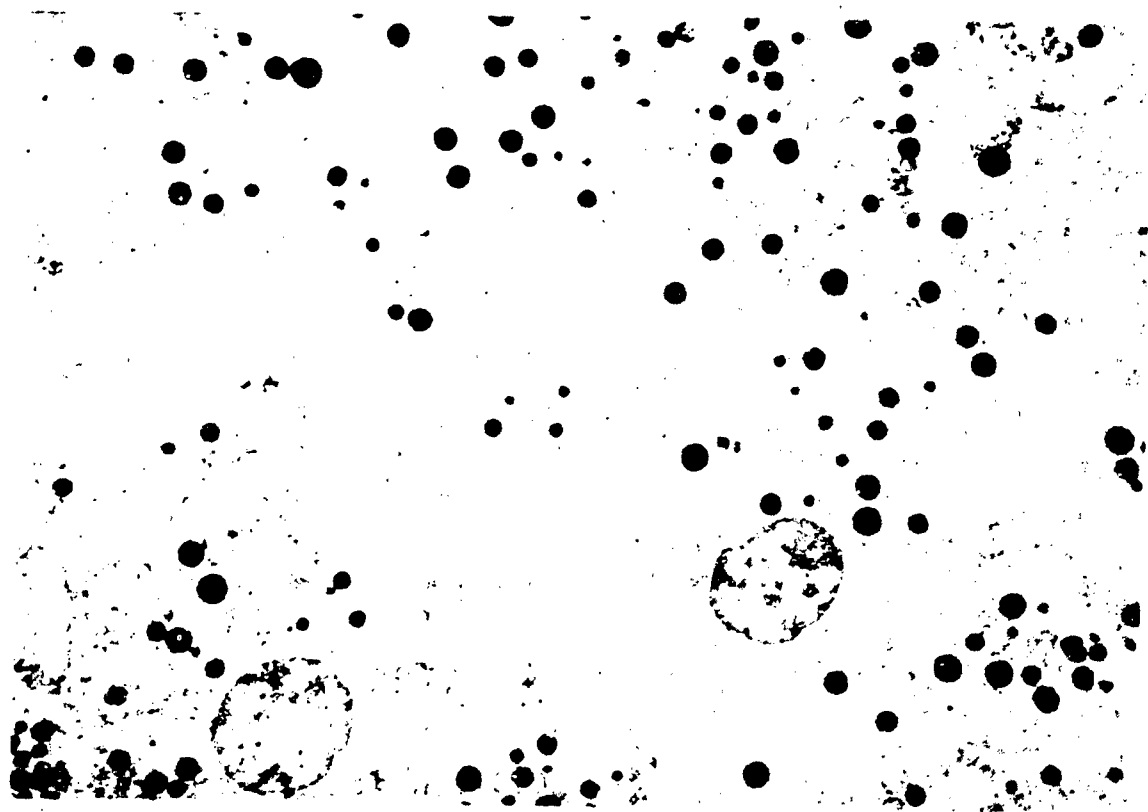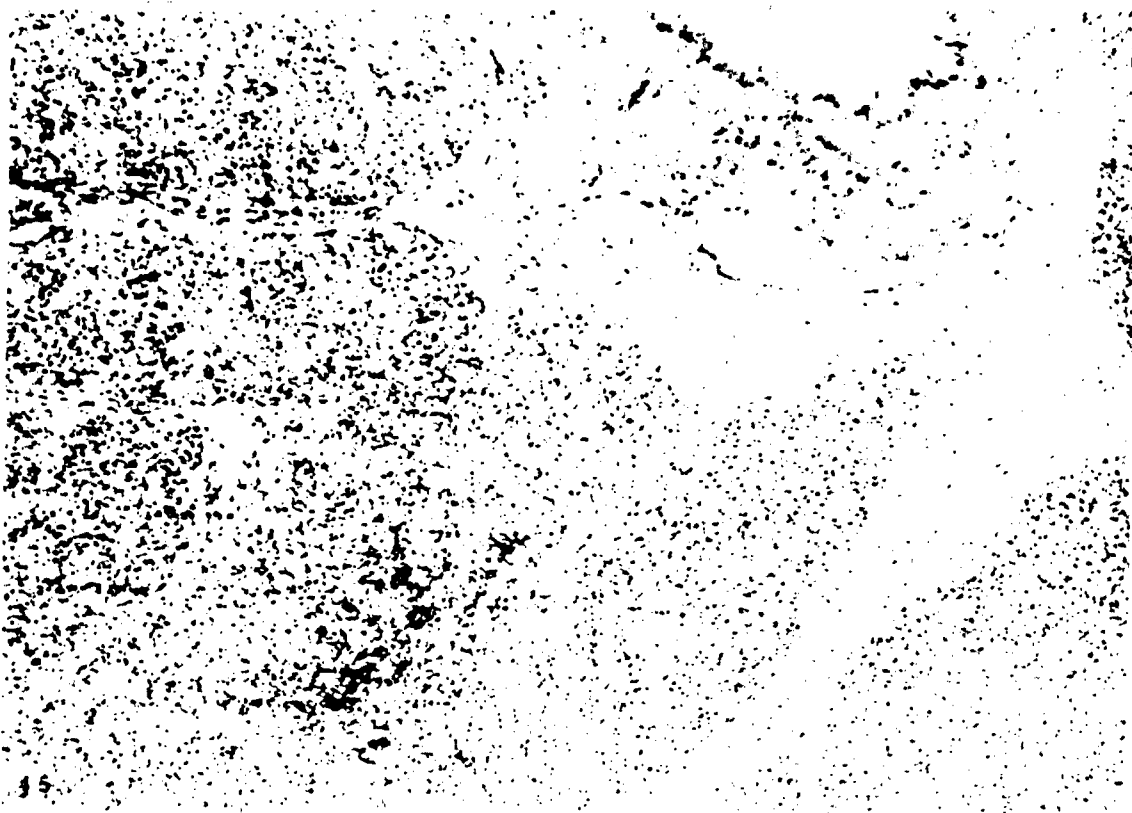
Fig. IIIA17 Pancreatic acinar cells of a pig given T-2 toxin (0.6 mg/kg) intravenously and killed 24 hours later. There is variable distension of the rough endoplasmic reticulum and perinuclear cistern which eventually disrupt and coalesce to form large membrane bound spaces. The nuclei of affected cells are pushed eccentrically and become necrotic, 32000 x.
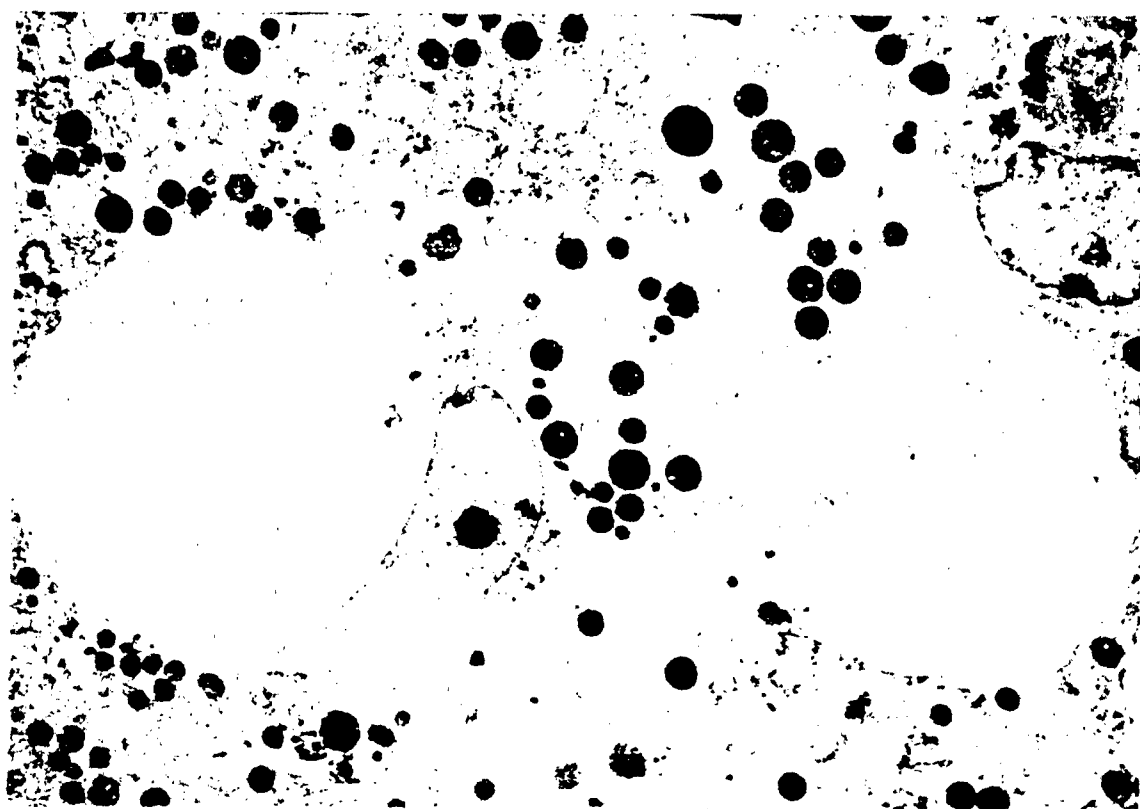
B. Additional Ultrastructural Studies in T-2 Toxin IV-Dosed Swine

OBJECTIVES

1) To identify the initial ultrastructural changes induced by T-2 toxin, particularly in reference to the myocardium.

2) To determine what organelles are most severely affected.

3) To determine the progression of these changes in acute toxicosis.

4) To determine the pathogenesis of these lesions and, therefore, of T-2 toxicosis.

PRELIMINARY STUDIES

In the first study, two pigs received 2.4 mg/kg T-2 toxin intravenously, a third pig was treated with vehicle alone, 50% ethanol intravenously, and a fourth pig served as an untreated control. This group of pigs was killed 30 minutes postdosing. No lesions were revealed in this group.

A second group of three pigs was similarly dosed, two receiving 2.4 mg/kg T-2 toxin intravenously, while the third served as a vehicle control. These pigs were killed 8 hours later. Target organs of T-2 toxin, such as lymphoid tissue of spleen, lymph node and thymus, as well as bone marrow, showed severe cellular degeneration and necrosis. Due to the severity of the lesions, progression of these changes could not be determined.

FORMAL STUDIES

The first formal study included eight, 8-week-old, male, castrated crossbred SPF derived piglets. T-2 toxin was given intravenously at doses of 0 (2 control pigs) and 2.4 mg/kg (6 treated pigs). Pigs were killed at 1/2 (1 T-2 pig), 1 (2 T-2 and 1 control pig), 4 (2 T-2 and 1 control pig), and 8 (1 T-2 pig) hours post-dosing. Sections from pancreas, spleen, splenic and bronchial lymph nodes, duodenum, ileum, colon, liver, heart, trachea and bone marrow were fixed by immersion in 2% glutaraldehyde in phosphate buffer.

Lungs were fixed by intratracheal instillation of fixative. Tissues have been processed and examination of semi-thin sections of the embedded tissue samples by light microscope has been carried out. Representative samples are presently being further processed for electron microscopic evaluation.

**Studies Remaining:**

The electron microscopic evaluation of the first formal study will be finished at the end of September, 1984. Further studies will be dependent on the findings of the first formal study.

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IV. METABOLISM OF T-2 TOXIN--Steven P. Swanson and Richard Corley

A. In Vitro Metabolism

OBJECTIVE 1 AND 2

To identify the major metabolites of T-2 toxin and to determine their rate of formation by using in vitro incubations with liver microsomal fractions. To employ in vitro incubation for production of metabolites.

METHODS

1. Microsomal incubation

Liver microsomes were prepared from male rats according to the method of Hansen et al (1981) discussed in detail on pages 42-56 of our first annual progress report. Conditions for incubation were as follows. Microsomes equivalent to 100 mg fresh liver were resuspended in 1 ml Tris buffer (pH 7.4 + 0.15 M KCl). Immediately prior to incubation the microsomal dilutions were quantitated for actual protein content according to standard laboratory methods. To 1 ml of microsomal preparation was added 3 ml of Tris buffer containing cofactors and 0.05 ml of either tritiated T-2 toxin or unlabeled toxin. The final concentration of toxin in the incubation mixtures was 0.536 mM for unlabeled T-2 and 0.0235 mM for tritium labeled T-2. Reactions were terminated at 5, 10, 15, 30 and 60 minutes by the addition of 0.5 ml of HCl.

In previous in vitro studies conducted in our laboratory with swine liver microsomes, reactions were quenched by adding 1g NaCl followed by partitioning of metabolites into ethyl acetate. Although this procedure efficiently extracted T-2 and HT-2, polar metabolites such as T-2 tetraol or conjugates were not quantitatively recovered. Extraction of reaction mixtures was therefore replaced with a more efficient C18 cartridge step. Incubation mixtures were loaded directly onto a C18 cartridge (preconditioned with 2 column volumes of methanol followed by two volumes of deionized water). The

cartridge was rinsed with 2 ml of water and the metabolites eluted with 2 x 0.9 ml of methanol, and the eluate concentrated to dryness. The residue was redissolved in 0.5 to 0.8 ml of ethanol for thin layer chromatographic and gas chromatographic analysis.

## 2. Analysis of incubation mixtures

Aliquots of extracts from tritiated T-2 toxin incubations with microsomes were analyzed by thin layer chromatography (both 1- and 2-dimensional). Extracts from incubation of cold T-2 were analyzed by gas chromatography and 2-dimensional thin layer chromatography.

One-dimensional TLC analysis of tritiated T-2 toxin-microsome extracts was accomplished by spotting aliquots on silica gel TLC plates (5x20 cm, 0.25 mm) along with appropriate trichothecene standards. The plates were developed in chloroform-methanol (9+1). Radiochromatographic profiles were obtained by scraping 2 mm wide bands from the TLC plates directly into scintillation vials. Two drops of water, 0.5 ml of methanol and 5 ml of Aquasol-2 were added and the radioactivity present was determined by liquid scintillation counting.

Two-dimensional high performance thin layer chromatography (2d-HPTLC) was also performed on extracts of microsomes incubated for 60 minutes with tritiated T-2 toxin. A 5 microliter aliquot of the extract was spotted on the corner of a Whatman HPK high performance TLC plate (10x10 cm, 0.2 mm). The plate was developed in the first direction using chloroform-methanol (9+1), air dried, then developed in the second direction (90 degrees to the first) in ethyl acetate-isooctane (3+1). Spots were visualized by spraying with methanolic sulfuric acid and charring.

Gas chromatographic analysis of reaction mixtures was accomplished by redissolving aliquots of extracts in 1.0 ml toluene and acetonitrile (95+5), adding 50 microliters of trifluoroacetic anhydride (TFAA) and heating 1 hour

at 60 degrees C in order to form the TFAA ester derivatives of T-2 and metabolites. After cooling, the mixture was washed with 1 ml of bicarbonate and the organic phase diluted with isooctane. One microliter was then injected into a capillary gas chromatograph under the following conditions: detector (ECD)-325 degrees, injector-250 degrees, flow (He)-35 cm/sec, temperature program-90(1)-170 @ 30 degrees/min, followed by a second temperature ramp of 170(1)-260 degrees @ 5.5 degrees/min. A 12mx0.2mm id bonded phase fused silica column was used.

Monitoring microsomal incubations by both thin layer radiochromatography and by gas chromatography were performed to utilize the advantages of each individual system. Incubations with tritium labeled T-2 toxin and monitoring by thin layer radiochromatography has the advantage of sensitivity. Very small quantities of radioactivity can readily be detected and metabolites found in only trace amounts can easily be counted. Although the tritium label on T-2 allows detection of trace amounts of metabolites, thin layer chromatography has a limited capacity for resolution of the metabolites. Metabolites very close in polarity may not be sufficiently resolved to be detected as distinct compounds.

Capillary gas chromatography, by comparison, is far superior to TLC in capability to resolve compounds of similar structure and polarity. The disadvantage of GLC is that only compounds which can be derivatized and volatilized will be detected. Together, both systems complement one another.

RESULTS

Results from microsomal incubations with tritiated T-2 toxin, which were analyzed by TLC after reaction times of 5, 10, 15, 30 and 60 minutes are given in Table IVA1. HT-2 was the major metabolite detected at all time peroids, however, the concentration of HT-2 did not exceed that of the parent T-2 until 30 minutes. In addition to HT-2, 3'-OH T-2 toxin, neosolaniol (Neo), T-2

tetraol (Tol), 4-deacetylneosolaniol (4-DN), and 3'OH HT-2 were also detected, with 3'OH T-2 toxin being the second most prevalent metabolite. As with previous incubations utilizing swine liver microsomes in our laboratory, the rat liver microsomes produced HT-2 very rapidly, within 5 minutes after addition of T-2 toxin.

The results of incubations with unlabeled T-2 toxin analyzed by capillary gas chromatography were similar although several minor differences were notable. HT-2 and 3'-OH T-2 were the two major metabolites detected. In contrast to incubations with tritium labeled T-2, neosolaniol and T-2 triol were also detected in trace amounts, however, T-2 tetraol and 3'-OH HT-2 were not detected (see Table IVA2). These minor variations in metabolite profiles between thin layer radiochromatography and capillary gas chromatography are most likely due to a) differences in initial substrate concentration and b) differences in sensitivity and resolution of the analytical methods as discussed in the methods section. Three additional peaks labeled RLM-1, RLM-2 and RLM-3 were observed, which increased in amount over time. The relative retention times of the three unidentified peaks were 0.8543 (RLM-1), 1.0180 (RLM-2) and 1.0913 (RLM-3), as compared to T-2 toxin. Further work on characterization of these compounds is in progress.

Analysis of extracts by two-dimensional HPTLC were similar to that obtained by GLC. Resolution was improved by the use of 2d-HPTLC over single dimensional TLC and by this method several new compounds were also detected. The new compounds detected by 2d-HPTLC and GLC were assumed to be metabolites of T-2 toxin, however, this has not been confirmed.

Further work on the in vitro metabolism of T-2 toxin is in progress. In addition to replication of the work described above, similar studies utilizing microsomal fractions from phenobarbital-induced rats have already been initiated.

One of the objectives of our _in vitro_ studies was to prepare metabolites for use as standards. This has not been necessary to date since standards were obtained by other means. However, many standards, particularly 3'-OH T-2 and 3'-OH HT-2 are available in very limited amounts. We anticipate that production of these two and possibly other metabolites, by _in vitro_ methods will be required within the next year.

Table IVA1

In Vitro Metabolism of Tritium Labeled T-2 Toxin by Male Rat Liver
Microsomes Over Time

| | % of Recovered Radioactivity | | | | |
| | Time in Minutes | | | | |
| Toxin | 5 | 10 | 15 | 30 | 60 |
| --- | --- | --- | --- | --- | --- |
| TOL | ND[a] | ND | ND | ND | 0.1 |
| 4-DN | ND | ND | ND | 0.7 | 0.9 |
| 3'-OH HT-2 | ND | ND | ND | 1.0 | 2.0 |
| HT-2 | 9.0 | 18.0 | 23.0 | 38.0 | 54.0 |
| NEO | ND | 0.4 | 0.5 | 2.0 | 0.7 |
| 3'OH T-2 | 5.0 | 9.0 | 12.0 | 15.0 | 17.0 |
| T-2 | 83.0 | 67.0 | 57.0 | 37.0 | 17.0 |

[a]
 ND -- none detected

Analysis of samples were determined by thin layer radiochromatography

Table IVA2

In Vitro Metabolism of T-2 Toxin by Male Rat Liver
Microsomes Over Time

| Toxin | Total Micrograms of Toxin Recovered Time in Minutes | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 15 | 30 | 60 |
| Neo | 1 | 2 | 2 | 2 | 3 |
| T-2 triol | 0.6 | 1 | 1 | 2 | 2 |
| HT-2 | 90 | 144 | 204 | 372 | 602 |
| T-2 | 727 | 547 | 563 | 537 | 330 |
| 3'-OH T-2 | 6 | 12 | 14 | 20 | 31 |

Quantitation was accomplished by capillary gas chromatography of
the corresponding trifluoroacetyl derivatives.  See text for
details.

## B. In Vivo Metabolism of T-2 Toxin in Swine

### OBJECTIVE 3

To administer tritiated T-2 toxin to swine and monitor radioactive profiles in blood, urine, feces, tissues and bile.

### METHODS

Reference Standards: Tritium-labeled T-2 toxin (radiopurity > 99%, specific activity 1.287 mCi/mg) was synthesized by the method of Wallace et al, 1977. Unlabeled standards of T-2, neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol and T-2 tetraol were produced in our laboratory. Additional standards of 3'-OH T-2 and 3'-OH HT-2 were obtained from another laboratory.

Animal Treatment: Two 20kg crossbred swine (Yorkshire x Hampshire; Thrushwood Farms, Fairbury, IL) were injected with erysipelas bacterin and acclimated to the large animal holding facility. All feeds offered to experimental swine were free from detectable concentrations of trichothecene mycotoxins and aflatoxins. Following preanesthetic administration of atropine sulfate, anesthesia was induced and maintained using halothane and oxygen. Indwelling catheters for dosing and blood collection were surgically implanted in the aorta via the femoral artery and were tunneled subcutaneously anterior and dorsal to the pelvis. The swine were returned to holding pens and allowed to recover for at least three days following surgery.

Immediately prior to dosing, HCl lidocaine was used as a local anesthetic and catheters were exteriorized via small skin incisions. Foley catheters were inserted into the urinary bladder. Tritium-labeled T-2 toxin was diluted with nonradioactive T-2 toxin in 1.5 ml of 50% ethanol such that each swine received one millicurie of total radioactivity at a dosage of 0.15mg T-2 toxin/kg body weight intravascularly as a single bolus injection. The route of administration and dosage level were selected for several reasons.

Previous work indicated that swine are particularly sensitive to the emetic action of T-2 toxin. Use of the intravascular route of administration insured total bioavailability of the toxin and allowed a comparison to toxicokinetic studies in similarly dosed swine. This non-lethal dose was also chosen to avoid the deleterious effect of T-2 toxin on urine output.

During the course of the experiment, the animals were restrained in a plastic-lined sling. Blood was collected in heparinized tubes and centrifuged to obtain plasma. Urine was collected hourly. Feces were collected as excreted. Although both swine exhibited periodic salivation, licking and chewing, all of which typically precede emesis in this species, vomition did not occur. The animals were euthanized four hours after toxin administration by inducing anesthesia with pentobarbital followed by exsanguination. Selected tissues and feces were weighed, flash-frozen in dry ice-isopropanol and stored at -20 degrees C prior to analysis. Sections of the gastrointestinal tract were separated from their contents and frozen.

Determination of Total Radioactivity: The total radioactivity in plasma and urine was determined immediately after collection. A 0.2ml aliquot of plasma or urine was added in duplicate directly to 5ml of Aquasol-2 liquid scintillation cocktail. The counting of radioactivity was performed with a Packard Tri-Carb 300M liquid scintillation counter. All data were corrected for background, dilution, quenching and counting efficiency.

RESULTS

Fig. IVB1 shows the total concentration of T-2 toxin and metabolites (total radioactivity) in blood plasma from these two swine (S1 and S2) over time. Total concentration of T-2 and metabolites dropped to less than 100 ppb by 2 hours and less than 40 ppb at the time of euthanasia The estimated mean plasma elimination phase half-life was 90 minutes for T-2 and metabolites (total radioactivity) in comparison to 13.8 minutes for the parent compound

alone, as reported in previous toxicokinetic studies in our laboratory (Beasley et al., 1984, see Appendix C).

A significant portion of the administered dose, 18.2% (S1) and 46.8% (S2), was excreted in the urine by the two pigs within 4 hours. Peak urine concentrations determined as total radioactivity (28 and 30 micrograms/ml) were reached in samples collected between 60 and 120 minutes. Urine data is summarized in Table IVB1.

Bile: The total radioactivity in bile was determined by incubating 0.02 ml of bile in 0.1 ml of 30% $H_2O_2$ at 60 degrees C for 1 hour. The samples were counted in 5ml Aquasol-2. A total of 14.1% (S1) and 1.3% (S2) of the administered dose was present in the bile of the two swine at 4 hours after dosing. The total amount of radioactivity excreted was related to the amount of bile retained. The gall bladder from pig S1 contained 13.3 ml of bile versus 3.9 ml for pig S2. Total concentrations for T-2 and metabolites (total radioactivity) in bile were 27 and 10 mg/ml for pig numbers S1 and S2, respectively.

Tissues: The total radioactivity in tissues, GI tract and contents and feces were determined by homogenizing 1 part sample in 4 parts water. A 0.05 ml volume of the homogenate was incubated with either 0.2 ml (tissues) or 0.3 ml (GI contents, feces) of Protosol tissue solubilizer at 60 degrees C for 2 hours. The samples were then decolorized by adding 0.1 ml 30% $H_2O_2$ followed by heating an additional 30 minutes at 60 degrees C. The samples were counted in 5 ml Aquasol-2.

Tissue residue levels of T-2 and its metabolites (total radioactivity) for animals S1 and S2 respectively were as follows: kidney (63,67 ppb), liver (32,30 ppb), mesenteric lymph nodes (35,27 ppb), spleen (23,25 ppb), muscle (16,23 ppb), lung (17,20 ppb), heart (14,19 ppb) and adrenal gland (16 ppb (S2 only)). The total radioactivity present in these tissues accounted for less

than 5% of the administered dose in both swine (see Table IVB2). The carcasses from both swine have been saved and additional samples such as brain, pancreas and bone marrow may be analyzed at a later date.

A total of 18.9% (S1) and 32.0% (S2) of the administered dose was found in the GI tract including contents of both swine. Results are summarized in Table IVB3. The presence of radioactivity in the different sections of the GI tract corresponds to the lesions reported in intravascularly-dosed swine. The ileum, which had the most severe lesions, contained the greatest amount of toxin (12% and 19% of the administered dose) respectively for S1 and S2) and the duodenum, with no apparent lesion, much less toxin (less than 0.3% of the administered dose). Less than 0.08% of the administered dose was detected in the feces of S1.

In summary, distribution data indicates that the GI tract, urine and bile are the only samples that contain appreciable concentrations of T-2 and its metabolites in swine 4 hours following IV administration of a sub-lethal dose. All tissues samples contained some radioactivity ranging in concentration from 14 to 67 ppb (total T-2 plus metabolites). The significance of these low levels cannot be assessed at this time. The presence of such a significant portion of the dose in the GI tract, however, strongly supports the use of adsorbents such as activated charcoal in the management of T-2 toxicosis. These data also support the theory that T-2 and its metabolites are eliminated adsorbents such as activated charcoal in the management of T-2 toxicosis. in 2 phases. First, a rapid clearance by the kidneys into the urine and by the liver into the bile. The second phase results from a slower release of metabolites into the GI tract itself. The effectiveness of bile as a means of excretion is dependent upon the ability of gut microflora to hydrolyze conjugates. Enzymatic hydrolysis of glucoronide conjugates by bacterial B-glucuronidase would free T-2 toxin and metabolites which could then be

reabsorbed by the intestinal walls with potential enterohepatic circulation. Additional studies with swine are needed to address this hypothesis.

OBJECTIVE 4

Identification of metabolites of T-2 toxin in IV-dosed swine.

METHODS

Urine and Bile: A 0.2 ml volume of urine or 0.02 ml of bile was added in duplicate to 2.0 ml acetate buffer (0.1 $M$, pH 3.8) and heated at 90 degrees C for 10 minutes to inactivate enzyme inhibitors. After cooling, one ml of either acetate buffer or B-glucuronidase (Sigma Chemical Co., type L-II from limpets; 3000 units/ml in 0.1 $M$ acetate buffer) were added to duplicate samples and incubated in a Dubnoff metabolic shaker at 38 degrees C for 18 hours. An additional B-glucuronidase incubation mixture containing phenolphthalein glucuronide was included with each set of samples to serve as a positive control. The samples were then added to a 500 mg C18 cartidge (preconditioned with two column volumes of methanol followed by two column volumes of deionized water). The sample tubes were rinsed 3x1 ml with deionized water and the rinses were added to the column. Metabolites were eluted with 2x0.8 ml methanol and concentrated to 0.5 ml (urine) or 0.2 ml (bile) for TLC radiochromatography. The extraction efficiency of radioactivity in urine and bile (Mean + SD) was 97.98 + 9.024%.

Plasma: With such a rapid decrease in the concentration of total radioactivity in plasma over time, a large sample volume (4 ml) was necessary for detection and identification of metabolites. Each sample, analyzed in duplicate, was added to 2.0 ml of acetate buffer. The pH was adjusted to 3.8 with glacial acetic acid (approximately 0.1 ml). To each duplicate sample, 2.0 ml of either acetate buffer or B-glucuronidase (4000 units/ml in 0.1 M acetate buffer) were added and all samples were incubated under the same conditions as urine. Due to the large amount of protein in plasma, 500 mg C18

cartidges were not used for extraction of metabolites. Instead, 10 ml of saturated NaCl were added and each sample was extracted 4 times with 20 ml of ethyl acetate. Centrifugation was necessary between each partition. The extracts were combined and filtered through a funnel containing 2 g $CaCO_3$ (basic) sandwiched between 2 layers of anhydrous sodium sulfate. The funnel was rinsed with an additional 20 ml of ethyl acetate and the extracts were concentrated *in vacuo*. The residue was redissolved in 0.1 ml toluene-acetonitrile (95+5) for TLC radiochromatography.

*Tissues, GI Tract and Contents*: To each of the homogenates prepared in the determination of total radioactivity, 5.0 g of NaCl was added and each sample was extracted 4 times with 20 ml of ethyl acetate. Centrifugation was necessary between each partition. The extracts were combined in a 250ml flask with 10g of anhydrous $NA_2SO_4$. To each flask, 2.5g $CaCO_3$ (basic) was added, filtered and concentrated. Samples were transfered to test tubes with 2x2 ml hexane followed by 4x2ml acetonitrile-methanol (1+1). The samples were shaken, centrifuged and the hexane layer discarded. The acetonitrile-methanol layer from tissue samples was concentrated to dryness at this point. Gastrointestinal tract and contents required an additional partition against hexane prior to concentration. Each sample was redissolved in 2 ml methylene chloride and added to a silica Sep Pak cartridge (preconditioned with 5 ml methylene chloride). The sample tubes were rinsed an additional three times with 2 ml methylene chloride and added to the cartridge. The cartridge was rinsed with 10 ml of methylene chloride-acetone (97+3) and metabolites were eluted with 15 ml methylene chloride-methanol (9+1) and concentrated. Tissue samples were redissolved in 0.1 ml toluene-acetonitrile (9+1) and GI samples in 0.2 ml methanol for TLC radiochromatography.

*TLC Radiochromatography*: Aliquots of each sample were spotted onto the outer channels of a precoated silica gel TLC plate (5x20 cm, 0.25 mm gel

thickness, J.T. Baker; scored into three equal channels) and standard compounds were spotted in the middle channel. The plates were developed in chloroform-methanol (9+1). To obtain radiochromatographic profiles of each sample, 1-2 mm wide bands were scraped from the TLC plates directly into scintillation vials containing 2-3 drops of water. A 0.5 ml volume of methanol was added and each sample was counted in 5ml of Aquasol-2. After scraping sample zones the remainder of the plates were sprayed with 30% $H_2SO_4$ in methanol and heated at 120 degrees C for 3-5 minutes. The standards were visualized under a long wave (365 nm) UV lamp. This method, although tedious, is very sensitive. Less than 30 dpm can be readily detected resulting in detection limits as low as 0.05 ppb.

Two Dimensional HPTLC: To confirm the presence of metabolites identified by TLC radiochromatography, two-dimensional high performance thin layer chromatography (2d-HPTLC) was used. After co-spotting each sample with standards in the corner of a precoated silica gel HPTLC plate (10x10 cm, 0.2 mm gel thickness, Whatman HP-K), the plates were first developed in chloroform-methanol (9+1). After air-drying, the plates were developed the second direction in ethyl acetate-isooctane (3+1). The compounds were visualized with p-nitrobenzylpyridine (p-NBP). This method provided complete resolution of all metabolites presently identified. Individual spots corresponding to known standards were scraped and analyzed for radioactivity to confirm the presence of metabolites identified by TLC radiochromatography. Unlike $H_2SO_4$, no loss of radioactivity occurs with the use of p-NBP.

RESULTS

Urine: A TLC radiochromatogram from a urine sample collected at 2 hours from pig S2 that was not incubated with B-glucuronidase is shown in Fig. IV B 2a. Free metabolites accounted for only 30% of the total radioactivity present in urine samples taken from both swine, with 3'-OH HT-2 as the

predominant metabolite. Seventy percent of the radioactivity present in urine samples was associated with very polar compounds that remained on the origin of the TLC plate. Hydrolysis of urine with B-glucuronidase yielded four major metabolites: T-2, 3'-OH T-2, HT-2 and 3'-OH HT-2 (see Fig IV B 2b) plus lesser amounts of neosolaniol, T-2 triol, 4-deacetylneosolaniol and T-2 tetraol. These 8 metabolites (free and conjugated) account for >90% of the total radioactivity in all urine samples. Original methods incorporating Amberlite XAD-4 provided excellent extraction efficiencies similar to C18 for free metabolites. Glucuronide conjugates, however, are not efficiently eluted from XAD-4. Replacement of XAD-4 with C18 was necessary when it was discovered that a majority of the metabolites were in the conjugated form.

Plasma: The major free metabolites identified, including the parent compound, were: T-2, 3'-OH T-2, HT-2 and 3'-OH HT-2. Other free metabolites detected at much lower concentrations included: neosolaniol, T-2 triol, 4-deacetylneosolaniol and T-2 tetraol. Unconjugated T-2 toxin and the major free metabolites (3'-OH T-2, HT-2, 3'-OH HT-2) accounted for 60% of the total radioactivity in plasma samples taken at 10 minutes after dosing but dropped to less than 10% by 4 hours. When duplicate samples were incubated with B-glucuronidase prior to extraction, an average of 89.2% of the total extractable radioactivity in plasma at all sampled time periods was associated with T-2, 3'-OH T-2, HT-2, and 3'-OH HT-2. Although 3'-OH HT-2 was the predominant free metabolite in plasma, 3'-OH T-2 and 3'-OH HT-2 were found to be nearly equal in concentration following enzyme hydrolysis. Concentrations of T-2 and its major metabolites in plasma are summarized in Fig. IVB3a-d. The percentage of T-2 and its major metabolites in the free and conjugated form are summarized in Fig. IVB4a-d. Although the metabolites were primarily in the conjugated form in plasma at 4 hours after T-2 toxin administration, the total concentration was still very low (< 40 ppb), indicating that blood

is not the sample of choice when diagnosing exposure to T-2 toxin.

Bile: Analysis of bile from pig S2 yielded a different metabolite profile than plasma or urine from the same animal (see Table IVB4). The major free metabolites ranging in concentration from 0.34 to 0.93 ppm were 3'-OH HT-2, T-2 triol, 4-deacetylneosolaniol and T-2 tetraol. Lesser amounts of T-2, 3'-OH T-2, neosolaniol and HT-2 ranging in concentration from .005 to .171 ppm were detected. Hydrolysis of bile with B-glucuronidase yielded the same four major metabolites identified in plasma and urine (T-2, 3'-OH T-2, HT-2, 3'-OH HT-2) but in different ratios. These metabolites (free and conjugated) accounted for >90% of the total radioactivity in the sample. Following enzyme hydrolysis, the concentration of T-2 toxin in all urine samples from S2 was less than the other major metabolites. The parent T-2 was by far the predominant compound in the bile, however, accounting for 49.8% of the total radioactivity, 1.6 times that of the other three major metabolites combined. Since T-2 is more toxic than its metabolites, this finding will be more significant if the gut microflora of swine are shown to hydrolyze the conjugates.

Tissues: Extractable free metabolites in tissues were composed primarily of T-2, 3'-OH T-2, HT-2 and 3'-OH HT-2. These metabolites account for 48% of the total radioactivity in mesenteric lymph nodes (MLN) and 27% in the spleen. In all other tissues less than 10% of the total radioactivity could be attributed to extractable free metabolites. The ratios of free metabolites varied with tissue type (See Table IVB5). Kidney contained predominately 3'-OH HT-2, while liver contained 3'-OH T-2 and HT-2 in addition to 3'-OH HT-2. The parent compound T-2, was not detected in liver or kidney by this method. In contrast, T-2, 3'-OH T-2, HT-2 and 3'-OH HT-2 were all detected in spleen, mesenteric lymph nodes (MLN), and heart. In all tissues the concentration of total free metabolites never exceeded 6 ppb, indicating that

the majority of radioactivity was associated with very polar or bound compounds.

To test the hypothesis that the radioactivity was associated with polar or bound compounds, samples of liver from both swine were treated with pancreatin, B-glucuronidase, NaCl, heat, pH manipulation and combinations of the above prior to extraction with ethyl acetate. Of these, pH manipulation plus NaCl and heat improved recoveries such that even the parent compound, T-2, which was not found previously in liver samples was detected. This suggests that protein binding (non-covalent) may be responsible for the low recoveries. Improvements in the tissue method incorporating these modifications is in progress. The determination of more accurate tissue profiles including free and bound metabolites will follow.

GI Tract: The same free metabolites present in other tissues and fluids have been qualitatively identified in the GI tract and its contents in addition to very polar nonextractable compounds that are most likely conjugates. Quantitation of these metabolites is in progress.

**Concentration (ng/ml) of T-2 and metabolites (total radioactivity) in plasma from two swine (S1 and S2) based upon the specific activity of the administered $^3$H-Labeled T-2 Toxin.**

Fig. IVB1
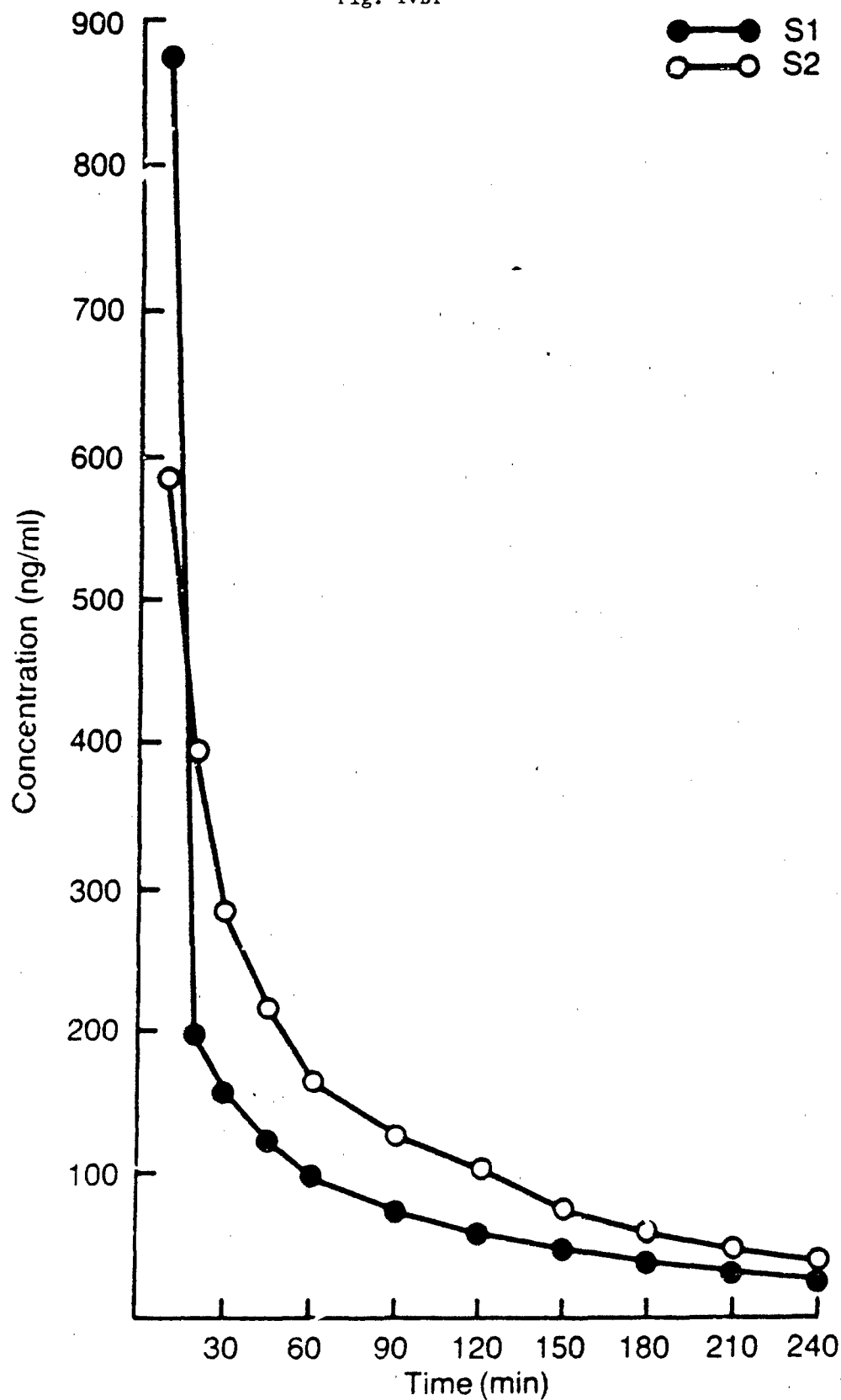
## Table IVB1

Concentration (ng/ml) of T-2 and metabolites (total radioactivity) in urine from two swine (S1 and S2) based upon the specific activity of the administered $^3$H-labeled T-2 toxin.

| Animal | Sample Time (min) | Total Volume (ml) | ug/ml | % of Dose Excreted | Cumulative % of Dose Excreted |
|--------|-------------------|-------------------|-------|--------------------|-------------------------------|
| S1 | 122 | 9.4 | 28 | 10.5 | 10.5 |
|  | 192 | 11.2 | 17 | 7.5 | 18.0 |
|  | 240 | 0.6 | 6 | 0.2 | 18.2 |
| S2 | 60 | 30 | 8 | 7.4 | 7.4 |
|  | 120 | 21 | 30 | 20.6 | 28.0 |
|  | 180 | 20 | 15 | 9.8 | 37.8 |
|  | 240 | 35 | 8 | 9.0 | 46.8 |

Table IVB2

Distribution of radioactivity in two swine (S1 and S2) at 4 hours
after administration of $^3$H-labeled T-2 toxin

| Sample | S1<br>% of Dose | S2<br>% of Dose |
|--------|------------------|------------------|
| Blood | 0.94 | 1.43 |
| Urine | 18.15 | 46.80 |
| Bile | 14.10 | 1.30 |
| Tissues | 3.57 | 4.83 |
| Liver | .52 | .51 |
| Kidney | .19 | .19 |
| Spleen | .03 | .03 |
| MLN | .06 | .06 |
| Lung | .11 | .13 |
| Muscle | 2.62 | 3.84 |
| Heart | .04 | .07 |
| TOTAL | 36.76 | 54.36 |

Table IVB3

Concentration (ng/g) of T-2 and Metabolites (Total Radioactivity) in the
Gastrointestinal Tract and Contents Collected at Time of Euthanasia (4 hr)
from Two Swine (S1 and S2) Based Upon the Specific ` tivity of the
Administered $^3$H-labeled T-2 Toxin

|  | S1 | | S2 | |
| --- | --- | --- | --- | --- |
|  | ng/g | % of Dose | ng/g | % of Dose |
| Stomach | 132 | 0.61 | 102 | 0.60 |
| Stomach Contents | 515 | 2.16 | 102 | 1.41 |
| Duodenum | 25 | 0.03 | 109 | 0.17 |
| Duodenal Contents | 43 | 0.02 | 142 | 0.07 |
| Jejunum | 218 | 1.56 | 191 | 1.78 |
| Jejunal Contents | 317 | 1.12 | 388 | 1.80 |
| Ileum | 714 | 4.21 | 452 | 4.54 |
| Ileal Contents | 1949 | 7.96 | 1869 | 14.32 |
| Lg. Intestine | 47 | 0.33 | 159 | 1.22 |
| Lg. Intestinal Contents | 93 | 0.85 | 214 | 6.05 |
| TOTAL | | 18.86% | | 31.96% |

Fig. IVB2a
Urine From Pig Number S2
Free Metabolites (no enzyme)
2.0 $\mu$l equiv. TLC Plate: 5 x 20 cm, 250 $\mu$m Si
Solvent: $CHCl_3$-MeOH (9 + 1)
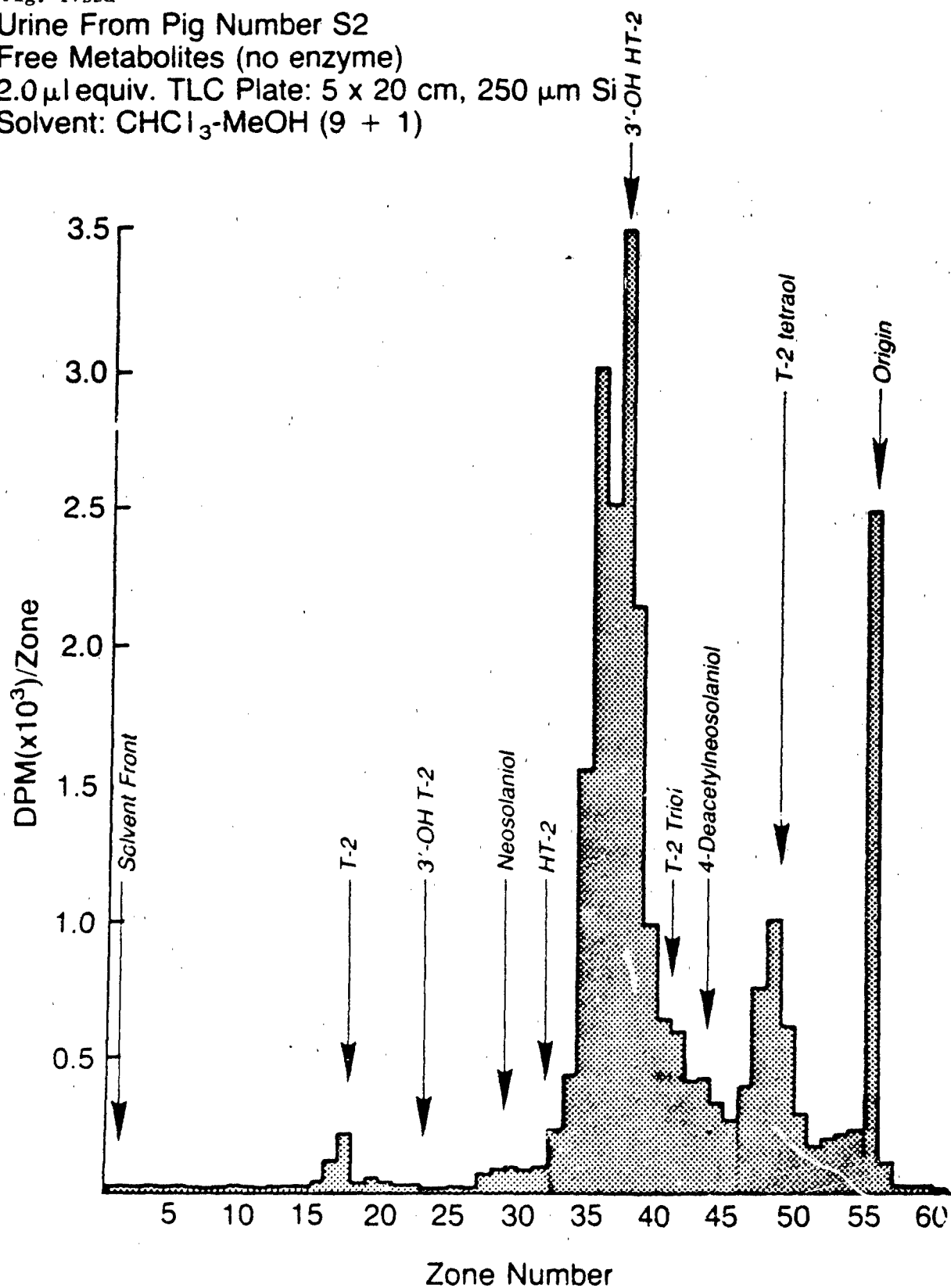
Fig. IVB2b
Urine From Pig Number S2
Total Metabolites (Free + Conjugates)
2.0 μl equiv.
TLC Plate: 5 x 20 cm, 250 μm Si
Solvent: CHCl₃-MeOH (9 + 1)
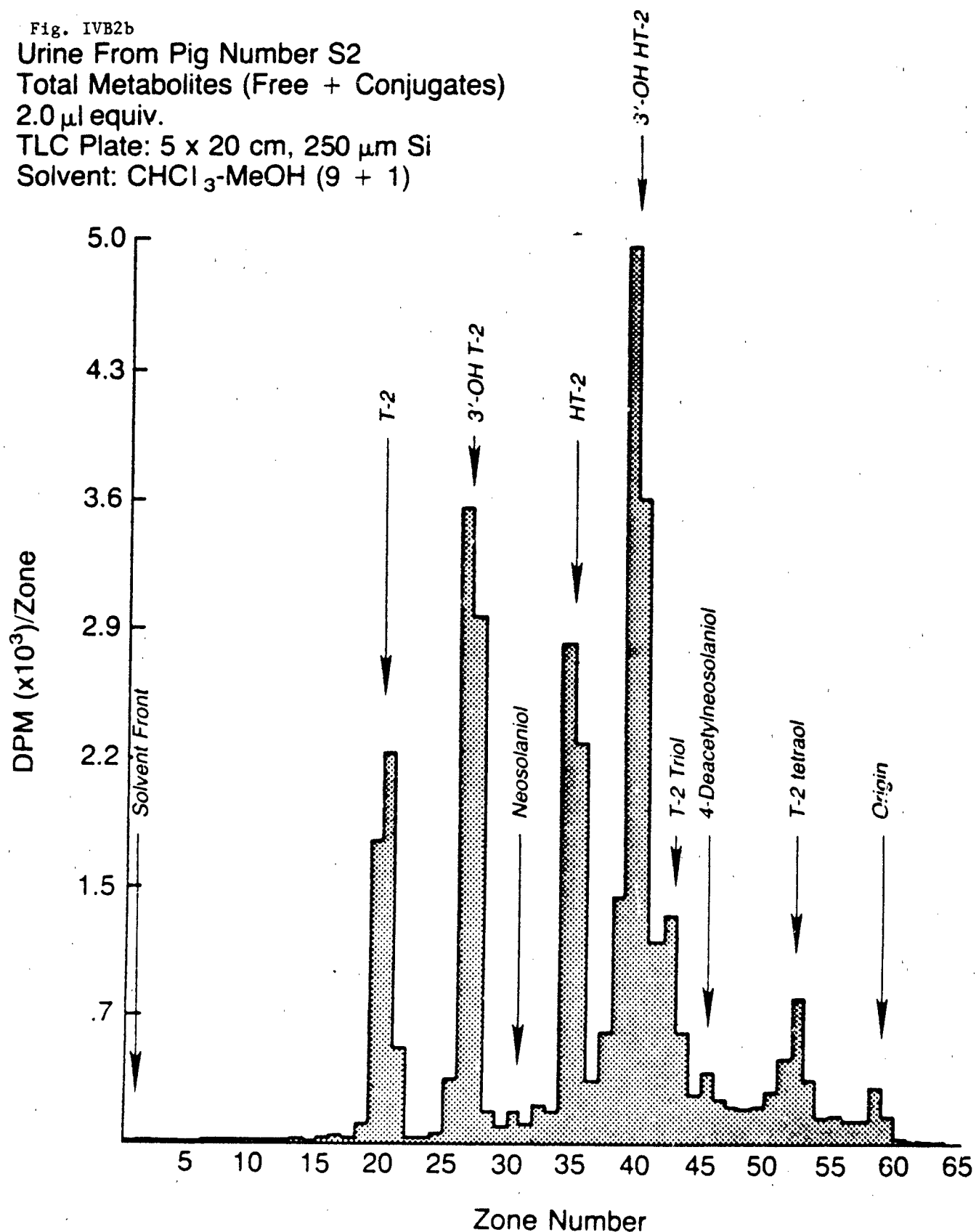
Fig. IVB3a  Concentration (ng/ml) of T-2 toxin in Plasma
from Pig Number S2 in the Free and Conjugated
Form .
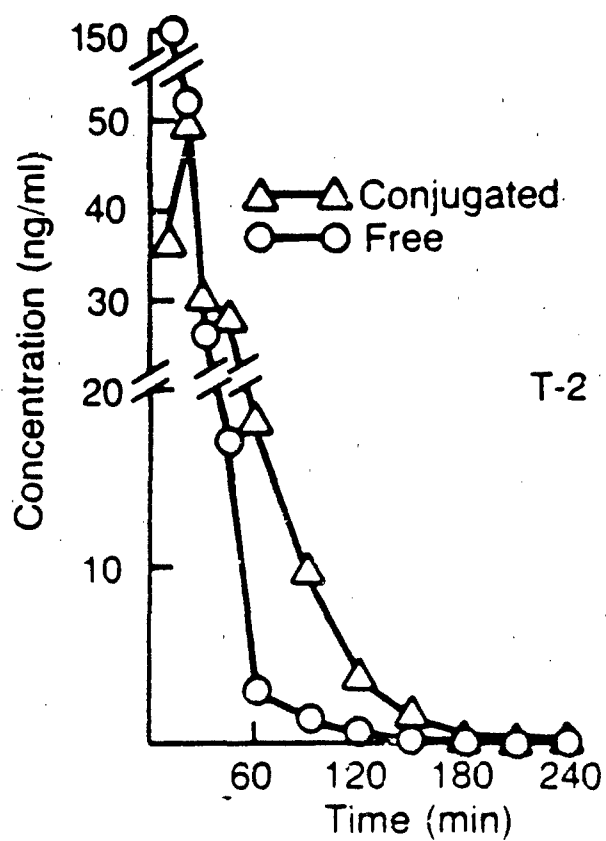
Fig. IVB3b  Concentration (ng/ml) of 3'-OH T-2 Toxin
in Plasma from Pig Number S2 in the Free
and Conjugated Form.
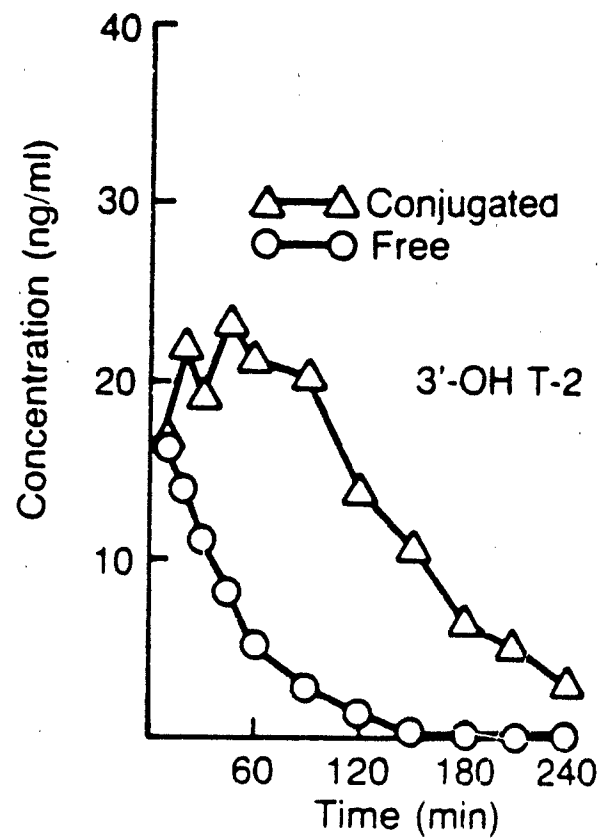
Fig. IVB3c   Concentration (ng/ml) of HT-2 Toxin in
Plasma from Pig Number S2 in the Free
and Conjugated Form.
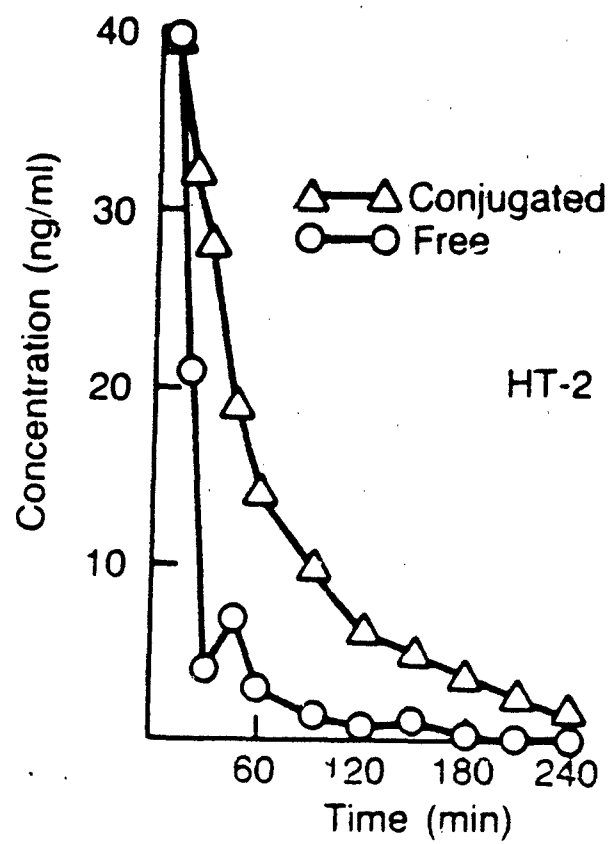
Fig. IVB3d   Concentration (ng/ml) of 3'-OH HT-2 Toxin
in Plasma from Pig Number S2 in the Free
and Conjugated Form.
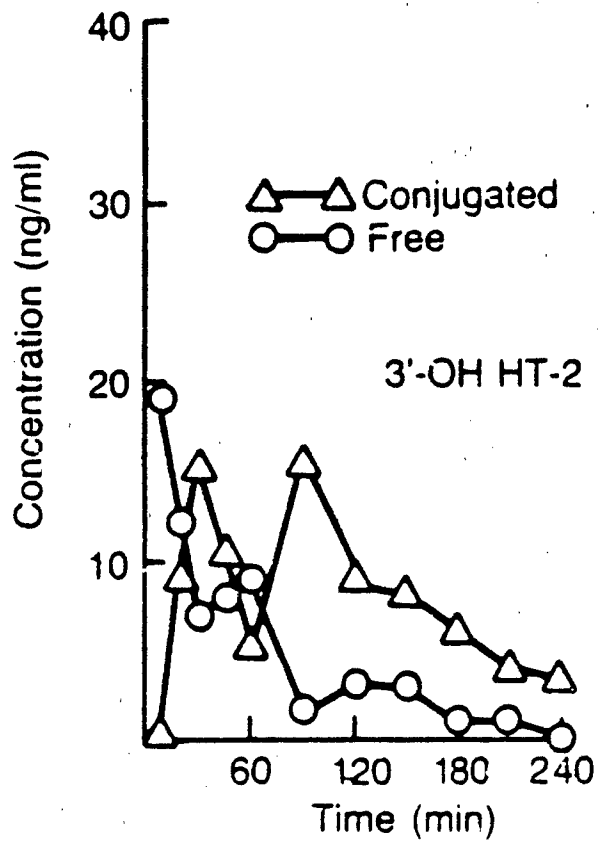
Fig. IVB4a    Percentage of T-2 Toxin in the Free and
              Conjugated Form in Plasma from Pig
              Number S2.
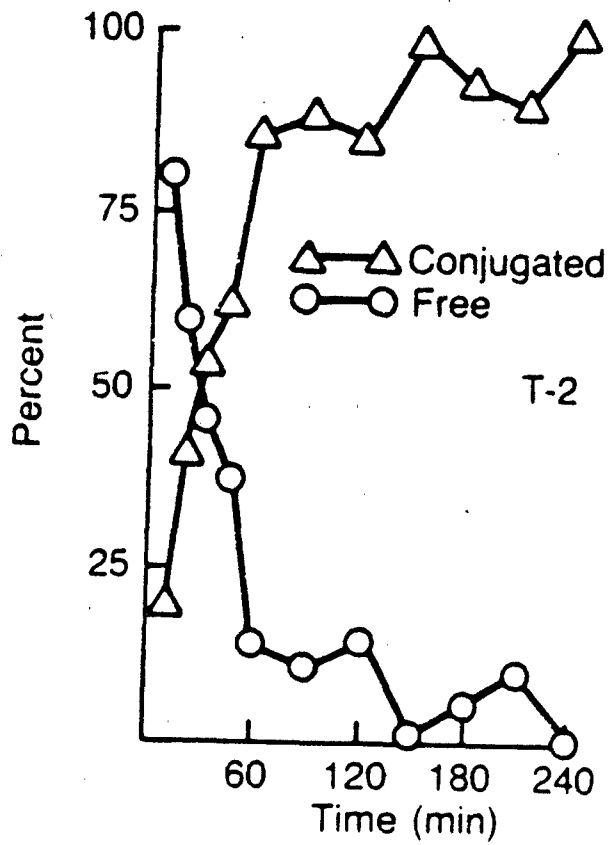
Fig. IVB4b   Percentage of 3'-OH T-2 Toxin in the Free
and Conjugated Form in Plasma from Pig
Number S2.
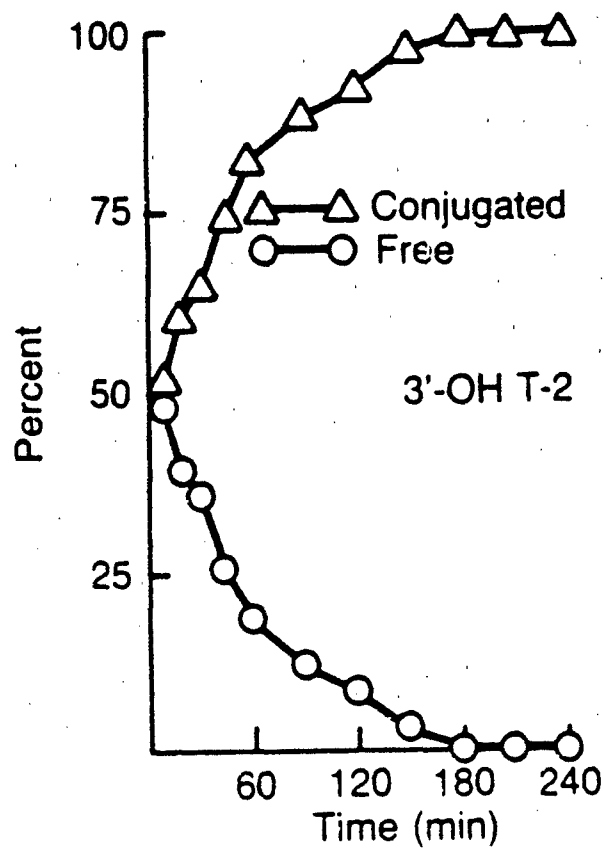
Fig. IVB4c   Percentage of HT-2 Toxin in the Free and
             Conjugated Form in Plasma from Pig
             Number S2.
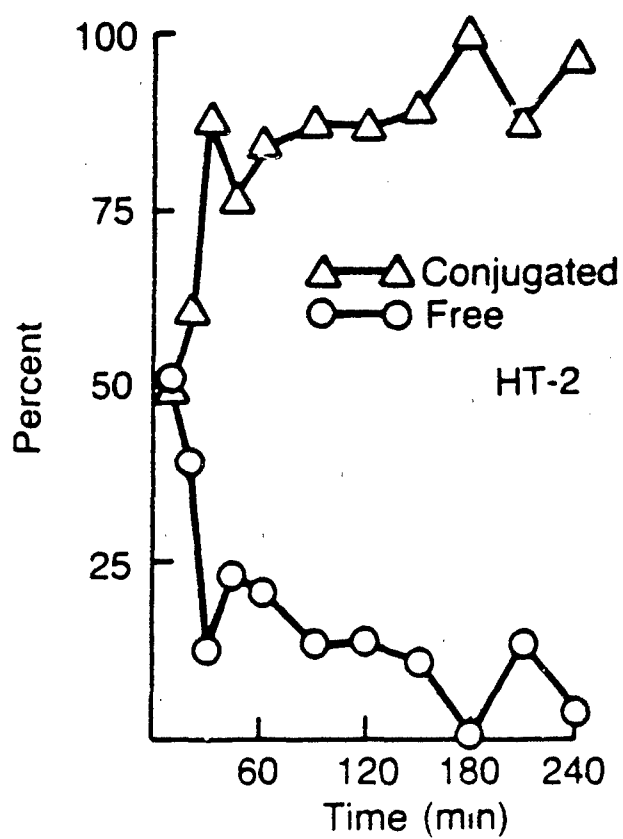
Fig. IVB4d  Percentage of 3'-OH HT-2 Toxin in the
Free and Conjugated Form in Plasma
from Pig Number S2.
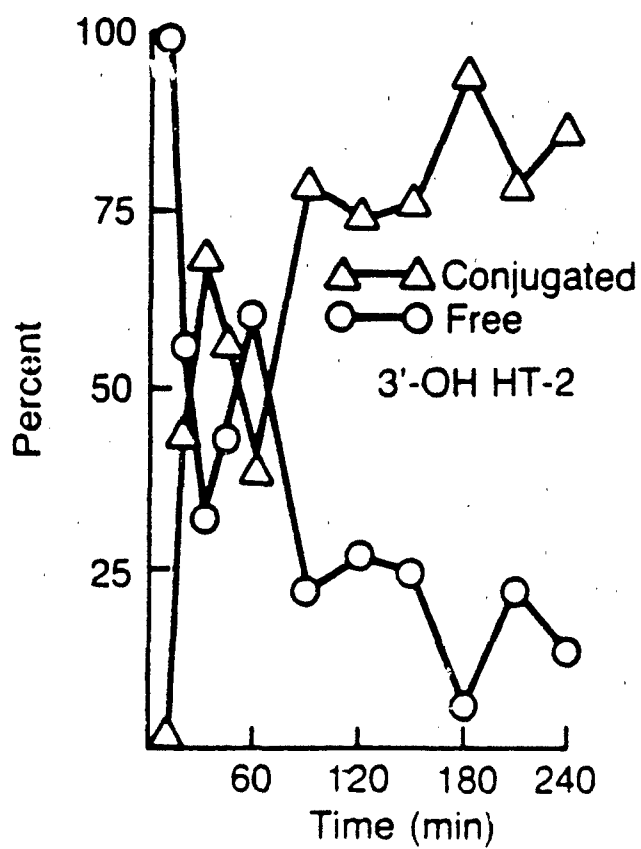
Table IVB4

Concentration (ng/ml) of T-2 and its metabolites in bile taken from pig number S2 collected at time of euthanasia (4h) in the free and conjugated form based upon the specific activity of the administered $^3$H-labeled T-2 toxin.

| | Free | Conjugated | Total |
|---|---|---|---|
| T-2 | 5 | 13,508 | 13,513 (49.81)[a] |
| 3'-OH T-2 | 25 | 1,287 | 1,312 (4.84) |
| Neo | 28 | 203 | 231 (0.85) |
| HT-2 | 171 | 5,011 | 5,182 (19.10) |
| 3'-OH HT-2 | 927 | 1,094 | 2,021 (7.45) |
| Triol | 518 | 302 | 820 (3.02) |
| 4-DN | 390 | 703 | 1,093 (4.03) |
| Tol | 336 | 73 | 409 (1.51) |
| Total | 2,400 | 22,181 | 24,581 (90.61) |

[a]
Number in parenthesis represents the percent of total radioactivity.

## Table IVB5

Concentrations (ng/g) of extractable free metabolites of T-2 toxin in tissues from two swine (S1 and S2) at time of euthasia (4h) based upon the specific activity of the administered $^3$H-labeled T-2 toxin.

| Tissue | T2 S1 | T2 S2 | 3'-OH T-2 S1 | 3'-OH T-2 S2 | Neosolaniol S1 | Neosolaniol S2 | HT-2 S1 | HT-2 S2 | 3'-OH HT-2 S1 | 3'-OH HT-2 S2 |
|---|---|---|---|---|---|---|---|---|---|---|
| Liver | ND[a] | ND | 0.29 | 0.75 | 0.25 | 0.12 | 0.84 | 0.71 | 2.66 | 1.06 |
| Kidney | ND | ND | <0.10 | ND | ND | 0.10 | 0.27 | 0.74 | 2.95 | 4.03 |
| Spleen | 1.64 | 0.90 | <0.10 | 2.12 | ND | ND | 0.86 | 1.71 | 1.33 | 1.97 |
| MLN[b] | 1.13 | 2.31 | 2.75 | 3.39 | ND | ND | 5.20 | 3.64 | 4.75 | 2.34 |
| Heart | <0.10 | 0.37 | <0.10 | 0.17 | <0.10 | <0.10 | 0.41 | 0.45 | 1.91 | 0.63 |

[a] ND indicates none detected

[b] MLN = mesenteric lymph nodes

OBJECTIVE 5

To prepare tritium-labeled deoxynivalenol.

RESULTS

Deoxynivalenol (100 mg) was sent to New England Nuclear Corporation for preparation of tritium-labeled toxin by the tritium gas exchange method. The resulting preparation of deoxynivalenol (1.1Ci) has been returned to the University of Illinois but has not yet been purified or assayed for specific activity.

OBJECTIVE 6

To determine the distribution and excretion of tritium-labeled deoxynivalenol in rats.

RESULTS

This work has not been started. Studies , scheduled for the end of 1984, are dependent upon the amount and specific activity of purified tritium-labeled deoxynivalenol which can be obtained from the crude radiolabeled preparation.

## V. RESIDUE ANALYIS/METHODS DEVELOPMENT

### OBJECTIVE 1

To improve methods for analysis of T-2 toxin and metabolites in biological samples.

### METHODS

Several methods were initially investigated for extraction of samples. Ethyl acetate partitioning and the use of Clin Elut extraction tubes efficiently extracted T-2, HT-2 and metabolites of similar polarity but were not efficient for the polar compounds such as T-2 tetraol. Reverse phase C18 cartridges provided efficient extraction of all known metabolites. The C18 cartridges, however, had a very limited sample capacity and although these cartridges worked well for metabolism studies with radiolabeled compounds where small sample sizes could be used, the sample capacity was insufficient for extraction of 5 or 10 ml volumes. Amberlite XAD-4 resin columns provided the best compromise between extraction efficiency and sample capacity and were therefore chosen.

The methods finally derived for analysis of blood, urine and bile are summarized below. Five to 10 ml of sample (bile or plasma were diluted 1+1 with water) and added to a 12 cm x 1 cm id column of XAD-4. The column was washed with 100ml water followed by 100 ml ethyl acetate-acetonitrile (9+1). The organic eluate was concentrated and the residue transfered to a 2.5 g column of florisil. The column was rinsed with 25 ml dichloromethane-acetone (97+3) and the trichothecenes eluted with 50 ml dichloromethane-methanol (9+1). After concentration the residue was redissolved in 5% aqueous methanol and added to a 500 mg C18 cartridge. The cartridge was rinsed with 1 ml water and eluted with 2 x 0.8 ml 85% methanol. The eluate was concentrated and redissolved in 1 ml toluene-acetonitrile (95+5) for derivatization.

Derivatization was accomplished by adding 0.05 ml heptafluorobutyryl-imidazole (HFBI) and heating 60 minutes at 60 degrees centigrade. After cooling the sample was washed with 1 ml sodium bicarbonate solution and an aliquot of the organic phase removed and diluted with hexane for gas chromatographic analysis.

Gas chromatography was performed by injecting 1 microliter of the derivatized extract into a capillary gas chromatograph with an electron capture detector. Conditions were as follows: detector-325 degrees C, injector-250 degrees C, column-12 m x 0.2 mm OV-1 bonded phase fused silica, dual ramp temperature program-90 degrees (hold 1 minute) to 180 degrees at 30 degrees /minute, 180 degrees (hold 1 minute) to 270 degrees at 7 degrees/minute.

RESULTS

The electron capture detector although very sensitive is not as selective as other detectors, therefore appreciable cleanup is required in order to eliminate sample interferences. Packed columns were previously used for analysis of T-2 residues. However, packed columns could not provide the necessary resolution of the numerous metabolites of T-2 toxin and still maintain sufficient sensitivity. We therefore switched to capillary gas chromatography where temperature programing could be utilized. This allowed greater separation of samples from interferences and also provided sufficient separation of individual metabolites while still maintaining sensitivity.

A chromatogram of trichothecene standards and a chromatogram of a swine plasma extract are given in Figure V1a and V1b.

Each individual trichothecene has a different detector response factor depending upon the number of free hydroxyl groups present in the molecule. Detection limits therefore vary depending upon the compound in question and the matrix. For clean preparations, such as plasma, the detection limits were 5-15 ppb. The detection limits for urine, which contains more sample interferences, were in the order of 10-30 ppb.
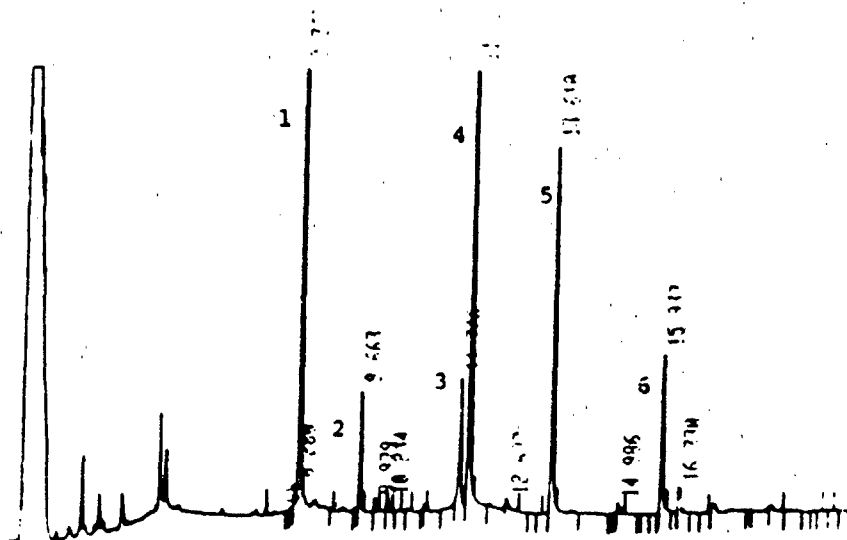
Fig. VIa  Gas chromatogram of trichothecene standards-HFB derivatives, 50-100
pg each.  1) T-2 tetraol, 2) 4-deacetylneosolaniol, 3) neosolaniol,
4) T-2 triol, 5) HT-2, 6) T-2.
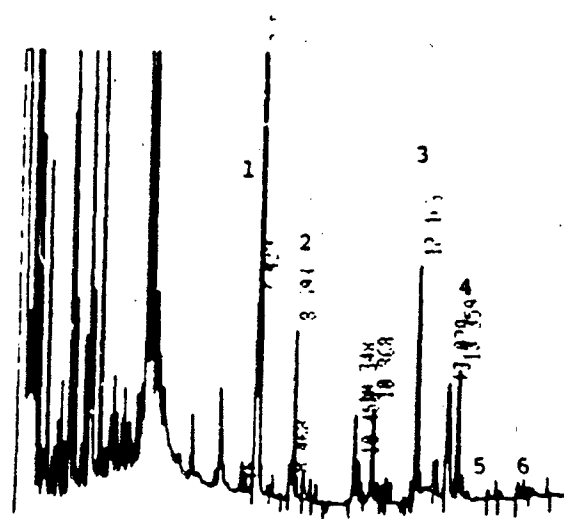
Fig. VIb  Gas chromatogram of a plasma extract from a swine administered
4.8 mg/kg T-2 toxin intravascularly.  1) T-2 tetraol,
2) 4-deacetylneosolaniol, 3) HT-2, 4) 3'OH HT-2, 5) T-2,
6) 3'OH T-2

OBJECTIVE 2

To determine residue concentrations of T-2 toxin and metabolites in swine dosed with T-2 toxin.

a) Dermally dosed swine (see Sections II and V, Objective 1 for Methods)

RESULTS

Samples of blood, urine, bile and tissues collected from the swine topically administered 15 mg/kg T-2 toxin in dimethyl sulfoxide have been analyzed by capillary GLC-ECD. All samples were collectd at thj time the animals were euthanized.

Neither T-2 toxin nor its known metabolites (as free compounds) were detected in the blood plasma or urine of the dermally-dosed swine when euthanized at 1, 3, 7 and 14 days post-dosing. In addition, no free T-2 residues were detected in bile.

Results in the previously described metabolism studies in swine, using tritiated T-2 toxin, indicated that glucuronide conjugates of T-2 and its metabolites were the major residues present in urine and plasma after the initial distribution phase. Therefore, selected samples of bile and urine from the dermally exposed swine were treated with B-glucuronidase to free conjugates. After extraction and cleanup the trichothecenes were hydrolyzed with potassium hydroxide to give T-2 tetraol followed by GLC-ECD anaylsis for T-2 tetraol. The bile from only one animal (euthanized 1 day post-dosing) contained toxin residues (as T-2 tetraol). Only two urine samples were positive (collected 14 days post dosing) after enzymatic hydrolysis to free conjugates followed by chemical hydrolysis to the parent alcohol T-2 tetraol. The finding of such residues after enzymatic hydrolysis indicates, however, that swine have the capacity to form glucuronides of T-2 (or metabolites) after topical exposure.

In contrast to the blood, urine, and bile, T-2 was detected in skin from all swine topically administered T-2 toxin. The mean concentrations in swine skin at the site of toxin application 1, 3, 7 and 14 days post dosing were 220, 247, 220 and 41 ppm, respectively. In addition to the parent compound, T-2 toxin, the following metabolites were detected in skin: HT-2, neosolaniol, 4-deacetylneosolaniol (4-DN), T-2 triol and T-2 tetraol. See Table V 2a for the mean concentration of T-2 toxin and metabolites in skin over time. The compounds 3'OH T-2 and 3'OH HT-2, major metabolites of T-2 in blood and urine of swine administered T-2 toxin intravascularly, were not detected in any of the skin samples from the dermally dosed swine.

Of particular interest was the finding that in general metabolites accounted for a very small percentage of the total toxin present in the skin at 1 and 3 days post dosing. In contrast, at 14 days post-dosing, metabolites represented nearly 40% of the total toxin residues presen in the skin. The most polar metabolite analyzed for, T-2 tetraol, was not detected in any skin samples at 1 day post-dosing and in only 25% of samples at 3 days post-dosing. However, T-2 tetraol was detected in all skin samples at 14 days post-dosing with a mean of 5.6 ppm.

Due to the variation in toxin concentration of skin samples from the same time period, it was decided to examine skin sections from two different swine in order to determine whether there was a concentration gradient across the skin lesions. One animal, euthanized 1 day post-dosing, and the second, euthanized 3 days post-dosing were selected. Skin specimens (approximately 10 g) were taken at the center of the lesion, near the edge of the visible lesion and just outside (0.5-1 cm) the visible lesion, (See Figure V2a). The results of the analysis are given in Tables V2b and V2c. In both animals the concentration of T-2 was the greatest at the center of the lesion and decreased in concentration at the edge of the lesion. It was interesting to

note that T-2 toxin was also detected in significant concentrations just outside the site of application where no skin lesions could be grossly observed. T-2 toxin was also detected in the subcutaneous fat immediately below the site of dermal application in all samples examined. The mean concentration of T-2 found in the fat is given in Table V2d.

The presence of T-2 toxin in the skin and fat of swine up to 14 days after topical administration indicates that T-2 is slowly absorbed by this route of administration. However, skin and fat do apparently act as a toxin reservoir. From a diagnostic point of view this finding demonstrates that the skin may serve as a potentially valuable sample source to confirm toxin exposure. Toxin concentrations within a visible lesion should be much greater than in blood or urine and therefore, chemical confirmation of dermal exposure would be much easier.

Table V2a
Concentration of T-2 Toxin and Metabolites in Skin of Swine at 1, 3, 7
and 14 Days after Topical Administration of T-2 Toxin at 15 mg/kg Body Weight

| | Concentration in ppm | | | |
|---|---|---|---|---|
| Toxin | a<br>1 day | b<br>3days | b<br>7 days | c<br>14 days |
| T-2 | 220±39 | 250±160 | 220±76 | 40±47 |
| HT-2 | 2.2±.47 | 2.6±1.1 | 57±59 | 6.4±2.8 |
| TRIOL | 0.06±0.02 | 0.21±0.06 | 7.4±12 | 8.7±15 |
| NEO | 0.90±0.78 | 1.2±0.57 | 12±4.4 | 0.45±0.24 |
| 1<br>4-DN | 0.73±0.32<br>d | 0.44±0.28 | 24±11 | 5.0±2.9 |
| TOL | ND | 0.02±0.04 | 2.2±1.1 | 5.6±7.9 |

a
 Mean ± SD of at least 3 animals
b
 Mean ± SD of 4 animals
c
 Mean ± SD of 5 animals
d
 None detected

Fig. V2a

LOCATION OF SKIN SECTIONS TAKEN FOR ANALYSIS

FROM SWINE TOPICALLY ADMINISTERED 15 MG/KG
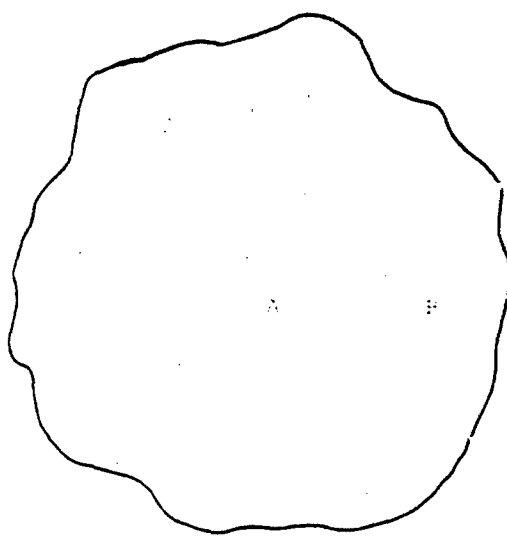
T-2 TOXIN.

Table V2b
Concentration of T-2 Toxin in Swine Skin Lesion 3 Days After
Dermal Administration of T-2 Toxin at 15 mg/kg

| LOCATION | T-2 (PPM) |
|---|---|
| Center of Lesion | 370 |
| Edge of Lesion | 157 |
| Center of Hemorrhage | 106 |
| Outside Lesion | 12 |

Table V2c
Concentration of T-2 Toxin in Swine Skin Lesion 1 Day After
Dermal Administration of T-2 Toxin at 15 mg/kg

| LOCATION | T-2 (PPM) |
|----------|-----------|
| Center of Lesion | 261 |
| Edge of Lesion | 180 |
| Outside Lesion | 54 |

Table V2d
Concentration of T-2 Toxin in Subcutaneous Fat of Swine 1, 3 and 14 Days After
Dermal Administration of T-2 Toxin at 15 mg/kg

| Days | T-2 Concentration (PPM) |
|------|-------------------------|
| 1    | 30 ± 5                  |
| 3    | 33 ± 26                 |
| 14   | 0.95 ± 0.21             |

Mean ± SD of 3 Animals

b) Residue analysis from swine administered 4.8 mg/kg T-2 toxin intravascularly.

METHODS

Blood and urine were collected at several time periods from swine administered 4.8 mg/kg T-2 toxin intravascularly. The samples were extracted and analyzed for free metabolites by capillary gas chromatography with electron capture detection as described above.

RESULTS

The concentration of T-2 toxin and metabolites detected in the blood plasma are given in Table V2e. The following metabolites were detected in the blood: T-2 tetraol, 4-deacetylneosolaniol (4-DN), neosolaniol (Neo), HT-2, T-2, 3'OH T-2, and 3'OH HT-2. T-2 toxin was detected in only one sample of plasma. Trace amounts, which could not be confirmed, were found in 3 additional samples. T-2 triol was not detected in any sample analyzed. The major metabolites detected were 3'OH HT-2, HT-2 and T-2 tetraol. In one animal (P221) significant amounts of neosolaniol were also detected.

Although these swine were administered a lethal dose of T-2 toxin (4.8 mg/kg), the residue levels of free metabolites in the blood were very low, with no individual metabolite ever exceeding 400 ppb.

The concentration of T-2 and metabolites in the swine urine is given in Table V2f. In addition to T-2, HT-2, 3'OH T-2, 3'OH HT-2, neosolaniol, 4-deacetylneosolaniol and T-2 tetraol, T-2 triol were also detected. In every sample the major free metabolite detected was 3'OH HT-2 and in many instances the concentration of 3'OH HT-2 exceeded by 100 times the concentration of the parent compound, T-2 toxin.

Results from both urine and blood analysis indicate that T-2 toxin is present in samples at very low concentrations. Extensive metabolism of the parent compound results in a variety of metabolites. From a diagnostic point

of view, it is clear that metabolites of T-2 toxin, especially 3'OH HT-2 are much better diagnostic indicators in blood, and that analysis for the parent T-2 toxin would be one of the worst possible choices for confirmation of toxin exposure. Analysis of blood for the parent compound T-2 could result in negative findings even in animals administered a lethal dose of T-2 toxin.

Table V2e
Concentration of T-2 Toxin and Metabolites in Blood Plasma of Swine
Administered T-2 Toxin Intravascularly at 4.8 mg/kg

| | | | | | | Compound in ppb | | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal # | Time [A] | Tol | 4-DN | Neo | Triol | HT-2 | 3'OH HT-2 | T-2 | 3'OH T-2 |
| | | | | B | | | | | |
| P215 | 2.0 | 116 | 22 | ND | ND | 148 | 356 | 20 | 40 |
| | | | | C | | | | | |
| | 4.0 | 142 | 15 | <5 | ND | 25 | 239 | ND | ND |
| | 6.0 | 146 | 15 | ND | ND | 23 | 249 | ND | ND |
| | 6.25 | 123 | 13 | ND | ND | 17 | 170 | ND | ND |
| P221 | 2.0 | 55 | 7 | 13 | ND | 10 | 60 | <5 | <5 |
| | 4.0 | 62 | 19 | 10 | ND | 19 | 37 | <5 | <5 |
| | 6.0 | 127 | 6 | 9 | ND | 19 | 37 | ND | ND |
| | 7.5 | 85 | 10 | 359 | ND | 23 | 50 | ND | ND |
| P217 | 2.0 | 104 | 17 | ND | ND | 118 | 149 | <5 | <5 |
| | 4.0 | 167 | 16 | ND | ND | 35 | 121 | ND | ND |
| | 6.0 | 147 | 14 | ND | ND | 22 | 167 | ND | ND |
| | 7.0 | 123 | 12 | ND | ND | 20 | 150 | ND | ND |

A
Time after dosing in hours
B
ND= none detected
C
Trace of toxin detected, concentration <5 ppb

Table V2f
Concentration of T-2 Toxin and Metabolites in Urine of Swine Administered
T-2 Toxin Intravascularly at 4.8 mg/kg

| | | Concentration in ppm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal | Time[A] | Tol | 4-DN | Neo | Triol | HT-2 | 3'OH HT-2 | T-2 | 3'OH T-2 |
| P213 | 1.5 | 0.21 | 0.05 | 0.22 | 0.09 | 1.3 | 23. | 0.15 | 2.3 |
| | 4.0 | 4.9 | 0.43 | 0.50 | 0.33 | 1.4 | 20. | 0.05 | 0.75 |
| | 5.25 | 3.8 | 0.39 | 0.60 | 0.26 | 0.86 | 20. | 0.10 | 0.50 |
| P215 | 2.0 | 0.54 | 0.70 | 2.6 | ND | 9.9 | 26. | 0.22 | 8.5 |
| | 4.0 | 16.0 | 3.3 | 2.2 | ND | 2.8 | 65. | 0.57 | 4.0 |
| P217 | 2.0 | 0.54 | 0.48 | 1.6 | 0.38 | 7.1 | 14. | 0.15 | 7.6 |
| | 4.0 | 1.4 | 0.85 | 1.2 | 0.29 | 2.0 | 13. | 0.04 | 2.8 |
| | 7.0 | 0.54 | 0.58 | 0.56 | 0.20 | 0.54 | 6.7 | ND | 1.5 |
| P221 | 2.0 | 4.3 | 0.85 | 0.45 | ND | 0.62 | 5.4 | ND | ND |

A
Time after dosing in hours

B
ND= None detected

## VI.  TRICHOTHECENE PRODUCTION/CHARACTERIZATION

OBJECTIVE 1

To collect isolates of Fusarium and screen these isolates for toxin profiles.

METHODS

Initial screening of fungi for toxin production was accomplished by growing the fungal isolation on corn or wheat followed by extraction with aqueous methanol. The aqueous methanol fraction was diluted with water and the trichothecenes partitioned into ethyl acetate or ethyl acetate-methylene chloride mixtures followed by gas chromatographic analysis of the extracts.

Although this method was not difficult, several disadvantages were apparent. First polar compounds such as T-2 tetraol were not efficiently partitioned into the organic phase after extraction. In addition, the extracts contained sufficient impurities to prevent detection of trichothecenes present in trace amounts.

A new method for screening fungal isolates was therefore investigated which utilized a modification of the vermiculite method described (Richardson, et al., 1984). Cultures were prepared by adding 1 g vermiculite, and 3.5 ml of glucose-yeast extract (GYE) medium to 20 ml vials. After autoclaving, agar plugs covered with mycelium were added and the vials incubated for 2 and 4 weeks at 25 degrees C. After incubation, the flasks were extracted twice overnight with acetonitrile. The extracts were combined, filtered and concentrated to dryness. The residue was redissolved in 0.5 ml ethanol for thin layer and gas chromatographic analysis.

A total of 10 isolates of Fusarium have been grown under the conditions described above, each in triplicate.

## RESULTS

A total of 10 isolates of _Fusarium_, 8 of _F. graminearum_ and 2 of _F. sporotrichioides_ were cultured, each in triplicate using the vermiculite system. Gas chromatographic analysis of the extracts has not yet been completed. Preliminary analysis by TLC revealed that 5 of the eight _F. graminearum_ cultures produced in excess of 50 ppm zearalenone. Only one isolate produced deoxynivalenol at a concentration of 3 ppm, and this isolate also produced zearalenone. Both _F. sporotrichioides_ isolates produced T-2 toxin at 30 and 40 ppm respectively.

Gas chromatographic analysis of the culture extracts and the screening of additional _Fusarium_ isolates will be completed by the end of 1984.

## OBJECTIVE 2

To prepare limited quantities of trichothecene standards by synthetic or biosynthetic routes.

## RESULTS

Neosclaniol, HT-2, T-2 triol, T-2 tetraol, acetyl T-2, and 4-deacetylneosolaniol, have been prepared from T-2 toxin by either chemical synthesis or as a biproduct of T-2 production via biosynthesis, extraction and purification. Additional standards of 3'OH HT-2 and 3'OH T-2 were obtained from another laboratory.

Although supplies of the above compounds were sufficient to meet current research needs, additional standards may need to be produced in the future in order to provide material for toxicological evaluation.

## OBJECTIVE 3

To inoculate young growing corn ears in the field with isolates of _Fusarium_ in order to produce nivalenol, fusarenone and deoxynivalenol.

RESULTS

No results. This objective was deleted at USAMRIID's request due to the potential political problems of inoculating corn fields with isolates of *Fusarium* which produce "Yellow Rain" toxins.

OBJECTIVE 4

To prepare T-2 toxin for large animal studies.

METHODS

Initial methods for production of T-2 toxin involved inoculating rice or wheat with *Fusarium*, incubating the grains for 4 weeks, followed by extraction, extensive cleanup and finally multiple recrystallizations. This resulted in T-2 toxin of high purity, however, the process was long and tedious due to the large amount of impurities coextracted from the solid medium.

In 1984, toxin production methods were changed to improve T-2 toxin production and purification. The current method involves growing *Fusarium* in flasks containing vermiculite which is coated with a glucose-yeast extract medium. With this medium, a relatively clean extract was obtained which required much less workup to give crystalline T-2 toxin. A detailed description of the growth and extraction parameters are given below.

The medium was prepared by placing 50 g vermiculite plus 105 ml glucose-yeast extract medium (150 g glucose, 0.5 g KCl, 0.5 g $MgSO_4$, 2g $NaNO_3$, 10 g $NH_4NO_3$, 1 g $K_2HPO_4$, 1 g yeast extract and 1 L deionized water) into 1 L erlenmeyer flasks. The flasks were autoclaved 15 minutes, inoculated with agar plugs covered with mycelium of *Fusarium sporotrichioides* NRRL 3299, previously named *F. tricinctum*, and incubated 24 days at 25 degrees C.

The cultures were broken into small fragments with a spatula and extracted 2 times with 400 ml ethyl acetate. The combined extracts were concentrated and purified, as follows:

1. Rapid Florisil column--dichloromethane-methanol (9+1)

2. Silica column dichloromethane-acetone (9+1)

3. HPLC ethyl acetate-isooctane (3+2)

4. Recrystallization

RESULTS

We presently have several batches of T-2 toxin in various stages of purification using the vermiculite method of toxin production.

Experiments with T-2 toxin using large animals such as swine for the animal model, require significant quantities of toxin. Inhalation experiments also require very large quantities of toxin since only a portion of the administered compound actually reaches the animal's lungs. Since the start of this contact (DAMD 17-82-C-2179, July 1982) a total of 32 g of T-2 toxin has been used for a variety of experiments including synthesis of metabolites and large animal studies employing intravascular, dermal and inhalation routes of administration. In several instances, especially for the inhalation experiments, a portion of toxin was recovered and recycled. During this time period, our laboratory has produced over 20 g of purified T-2 toxin. We are continuing to produce T-2 toxin to supplement that supplied by USAMRIID and thereby fulfill the toxin requirements of our group.

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APPENDIX A

Experimental T-2 Toxicosis in Swine. I. Changes in Cardiac Output, Aortic Mean Pressure, Catecholamines, 6-keto-PGF$_{1\alpha}$, Thromboxane B$_2$ and Acid-Base Parameters.[1,2]

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## ABSTRACT

Experimental T-2 Toxicosis in Swine. I. Changes in Cardiac Output, Aortic Mean Pressure, Catecholamines, 6-keto-PGF$_{1\alpha}$, Thromboxane B$_2$ and Acid-Base Parameters. Lorenzana, R.M., Beasley, V.R., Buck, W.B. (1984). Fundam. Appl. Toxicol. T-2 Toxin given as a single intravascular dose to swine produced a shock syndrome. Dosages of 0.6 or 4.8 mg/kg were administered to different groups of swine. Shock was characterized by reductions in cardiac output and blood pressure, and increased plasma concentrations of epinephrine, norepinephrine, thromboxane B$_2$ and 6-keto-PGF$_{1\alpha}$ and lactate. Total peripheral resistance was unchanged in the high dose group but decreased in the low dose group. Pulmonary vascular resistance increased in both groups. Decreases occurred in arterial pH and arterial oxygen partial pressure. No alterations occurred in plasma concentrations of histamine or serotonin.

# INTRODUCTION

T-2 toxin (3α-hydroxy-4β, 15-diacetoxy-8α-(3-methylbutyryloxy)-12, 13-epoxytrichothec-9-ene) is a secondary metabolite of several species of Fusarium. Trichothecene production by Fusaria tends to occur in grain over-wintered in the field and in high-moisture grains that have been improperly stored. Although T-2 toxin is not frequently detected in grains used for animal feed in the United States, when T-2 toxin is identified, it is associated with serious detrimental effects (Hsu et al., 1972; Greenway and Puls, 1976). As early as the 1940's when a widespread human disease called alimentary toxic aleukia (ATA) was recognized in the USSR (Forgacs and Carll, 1962), clinical syndromes in both man and animals were associated with the consumption of moldy grain. Fusarium producers of T-2 toxin were later found to be among the predominant fungi apparently responsible in that outbreak of ATA (Joffe and Yagen, 1977). Historically, Fusarium mycotoxins have been associated with moldy corn toxicosis in cattle in the USA, Akakabibyo (red-mold) disease in humans and animals in Japan, and bean hull poisoning in horses in Japan (Pathre and Mirocha, 1979; Saito and Ohtsubo, 1974; Ueno, 1977). Most controversial of the toxicoses produced by the trichothecenes involves their alleged use by communist forces in chemical weapons in Laos, Cambodia (Kampuchea) and Afghanistan. The popularized pseudonym, "Yellow Rain", refers to the yellowish cloud sprayed from planes or burst from shells in the form of sticky yellow drops. Reported effects include skin rashes, difficult respiration, emesis, hemorrhage and death within a brief period of time in humans, as well as death in exposed animals and plants (Schiefer, 1982). Samples taken from foliage revealed 48 ppm T-2 toxin and the presence of other Fusarium mycotoxins (Rosen and Rosen, 1982). HT-2, a metabolite of T-2 toxin was found in the blood of two alleged victims (Congressional special report, 1982).

In our previous work with swine given T-2 toxin intravascularly, it became apparent that the animals died following a shock syndrome induced by the toxin. At an intravascular LD50 dose (1.2 mg/kg), shock progressed to death within twelve to sixteen hours (Beasley, 1983; Weaver, et al., 1978).

Since the cardiovascular and pulmonary physiology of swine is very similar to that of man, and swine are of sufficient size to allow collection of multiple blood specimens, the pig is considered an appropriate model (Tenney and Remmers, 1963; Dodds, 1982). It was the purpose of this study to characterize the pathophysiology of acute T-2 toxicosis in swine. In this report, the changes in hemodynamic function, blood gases and pH, and vasoactive mediators in response to T-2 toxin given intravascularly are described.

## MATERIALS AND METHODS

### Chemical and animals

Purified T-2 toxin[3] was dissolved in 6.5 ml of 70 percent ethanol. White, crossbred female swine ranging from 40-60 kilograms were immunized against erysipelas,[4] given an intramuscular injection of selenium and allowed to acclimate to their housing and feed for four to seven days prior to surgical catheterization. After surgery, the animals were allowed to recover for two to four weeks, and were active and in good health at the time of the study. Swine were fed a commercial diet which contained no detectable concentrations of vomitoxin (deoxynivalenol), zearalenone, T-2 toxin, diacetoxyscirpenol (DAS), or aflatoxins B1, B2, G1 and G2.[5]

### Surgical preparation

Anesthesia was induced in fasted swine using 5 percent halothane in a mixture of oxygen and nitrous oxide via a nose cone. After endotracheal intubation, anesthesia was maintained with 0.75-1.0 percent halothane in a mixture of oxygen and nitrous oxide using a closed circuit system[6] and intermittent

positive pressure ventilation.[7] The heart rate and rhythm were monitored while a left lateral thoracotomy was performed. Non-compliant fluid-filled catheters[8] were implanted in the pulmonary artery and left atrium via pericardotomy. Another catheter was placed in the ascending aorta via the internal thoracic artery (Tranquilli et al., 1982).

These catheters were filled with heparin, exteriorized through the lateral thoracic wall and buried in the subcutis until the day of the study. Each animal was given antibiotics post-surgically, and the skin sutures were removed after ten days.

Experimental protocol

In a preliminary group, T-2 toxin was given intravascularly to evaluate procedural protocols, instrumentation, the appearance of the vasoactive substances histamine, serotonin, epinephrine, norepinephrine, and the hydrolysis products of thromboxane $A_2$ and prostacyclin, thromboxane $B_2$ ($TXB_2$), and 6-keto-$PGF_{1\alpha}$, respectively. In this group, two pigs received T-2 toxin at 5.4 mg/kg and one pig received T-2 toxin at 1.2 mg/kg.

In the formal study, pigs were divided into three groups. A lethal dose of 4.8 mg/kg, was given to five swine (high dose group), and a sublethal dose of 0.6 mg/kg was administered to five other pigs (low dose group). Five control swine received only the 70 percent ethanol vehicle intravascularly in addition to undergoing identical surgical and handling procedures.

On the day of the study, the conscious animal was restrained in a webbed stanchion which allowed the pig to rest on its sternum and abdomen while its legs hung below the webbing. All animals quickly adapted to this method of restraint and appeared to rest comfortably. After the overlying skin was

infiltrated with 2 percent lidocaine HCl, the catheters were exteriorized through small skin incisions and attached via manifolds to measuring and sampling devices.

Prior to dosing two blood samples were withdrawn from the aortic catheter for determination of arterial concentrations of oxygen ($PaO_2$) and carbon dioxide ($PaCO_2$), as well as arterial pH ($pH_a$). Multiple cardiac output determinations were made and aortic mean pressure was recorded prior to the collection of blood samples for baseline clinical chemistry determinations. The toxin solution was then administered into the pulmonary artery by a continuous infusion over a five-minute period.[9] The beginning of the infusion was considered to be the starting time of the experiment.

All catheters were frequently flushed with heparinized saline to ensure patency and the absence of clots in the samples. During the course of the experiment, each pig received four liters of fluid due to this flushing. The amount of blood taken for clinical determinations was less than 10 percent of the animal's total blood volume (i.e., five hundred milliliters or less).

In the high dose group, on the day before the study, a self-retaining catheter was passed into the urinary bladder. During the study, blood samples and hemodynamic measurements were taken up to the time of death. In the low dose and control groups, samples and measurements were made from predosing through 24 hours after toxin administration. The animals were then subjected to euthanasia by exsanguination after receiving an anesthetic dose of sodium thiamylal.

Hemodynamic measurements

Pressures in the left atrium, pulmonary artery and aorta were measured every thirty minutes using noncompliant fluid filled systems and transducers[10] on a multichannel physiograph.[11] Transducers were zeroed at the

level of the scapulohumeral joint which was considered to correspond to the level of the right atrium.

Cardiac output was determined every thirty minutes by an indicator dilution technique (Manohar, 1978). Indocynanine green[12] was injected into the left atrium and blood was withdrawn from the aorta at a known constant rate through a linear densitometer.[13] The resultant curve area was determined by semi-logarithmic plotting of the downslope on a computer.[14]

Total peripheral resistance (TPR) was calculated as the quotient of aortic mean pressure (AOM) divided by cardiac output (CO). Pulmonary vascular resis-tance (PVR) was calculated by subtracting the left atrial mean pressure (LAM) from pulmonary artery mean pressure (PAM) and dividing the difference by CO.

Clinical chemistry determinations

Arterial blood was anaerobically collected every thirty minutes from the aortic catheter for determination of $PaO_2$, $PaCO_2$, and $pH_a$.[15] Blood gas tensions and $pH_a$ were corrected to the animal's rectal temperature, using temperature coefficients published for human blood (Severinghaus, 1966).

Plasma lactic acid concentrations were determined from aortic blood. Blood was collected every thirty minutes in chilled syringes, immediately added to chilled perchloric acid and agitated in a vortex for thirty seconds. The pro-tein precipitate was removed by centrifugation, and the plasma was maintained for no longer than seven days at 4°C before analysis.[16]

Ten ml of aortic blood was collected hourly for catecholamine analysis in chilled, heparinized syringes, immediately transferred to chilled, heparinized glass tubes and gently mixed for fifteen seconds before being centrifuged at 2000 g at 4°C for ten minutes. The plasma was transferred to plastic vials and quick-frozen in a dry ice and alcohol bath. The samples were stored at -70°C until analyzed as previously described (Chou et al., 1983).[17]

Aortic blood was collected into chilled syringes every thirty minutes for the first two hours, and thereafter on an hourly basis for the determination of concentrations of 6-keto-PGF$_{1\alpha}$ and TXB$_2$. The blood was immediately added to siliconized glass tubes containing 2 percent EDTA and 0.4 percent aspirin in saline, gently mixed for fifteen seconds and then centrifuged at 2000 g at 4° for ten minutes. Plasma was decanted into plastic vials using plastic pipettes, and the samples were frozen and maintained at -20°C until analysis. The concentrations of 6-keto-PGF$_{1\alpha}$ and TXB$_2$ were determined by radioimmunoassay.[18] The procedure was previously validated for use with unextracted porcine plasma.

Duplicate samples were included in each assay for the concentrations of 6-keto-PGF$_{1\alpha}$ and TXB$_2$. If the coefficient of variation for the replicates was greater than twelve percent, the sample was rerun in a subsequent assay. Individual samples were frozen and thawed only once prior to assay and were assayed within a few hours of thawing.

The intra-assay coefficients of variation for TXB$_2$ and 6-keto-PGF$_{1\alpha}$, respectively, were 4.7 percent and 5.7 percent. The inter-assay coefficient of variation for TXB$_2$ was 7.4 percent for a high concentration pooled plasma sample and 3.8 percent for a low concentration sample. The inter-assay coefficient of variation for 6-keto-PFG$_{1\alpha}$ was 6.8 percent for a high concentration pooled plasma sample and 15.3 percent for a low concentration sample. The TXB$_2$ and 6-keto-PGF$_{1\alpha}$ recovery rates were 91.2 percent and 86.1 percent, respectively. The sensitivity limits of the assays were considered to occur at 90 percent binding and the mean values were 6.3 pg/ml for TXB$_2$ and 17.6 pg/ml for 6-keto-PGF$_{1\alpha}$.

Plasma serotonin and histamine concentrations were determined in the three preliminary swine. Blood for serotonin analysis was collected in chilled syringes, added to chilled plastic vials containing EDTA and ascorbic acid, and gently mixed for ten seconds. The samples were then centrifuged at 2000 g at 4°C for ten minutes, and the plasma was removed using plastic pipettes, placed in plastic vials and quick-frozen in a dry ice and alcohol bath. The samples were stored at -70°C until analyzed as previously described (Chou, 1983).[17]

Blood for histamine analysis was collected in chilled syringes, added to plastic vials containing potassium oxalate and gently mixed for ten seconds. The samples were then handled in a manner similar to the procedure for serotonin and the plasma was stored at -70°C until analyzed by a flurometric technique.[19]

Statistical evaluation

Widely differing predose values and postdose variances in these data negated standard analyses of variance methods. As an alternative approach, each animal in the three (High, Low and Control) groups was tested for significant upward or downward trend in each parameter, these trends being assessed for significance by both Pearson's r and Kendall's tau two-tailed correlation analyses, with the time sequence of successive observations serving as the independent variate. In this approach, all animals were individually tested against the hypothesis that treatment had no nonrandom effects upon the monitored parameters. Intergroup differences were then judged by comparing the sets of intragroup test with each other. In this way, for example, five high dose animals, all showing a significant increase in a particular parameter, were clearly behaving differently than a population of five low dose animals all showing either no change or the opposite change in the same parameter.

A value of P less than 0.05 was considered statistically significant. During the early time period when the high dose animals were alive, these intergroup comparisons were buttressed by the Kruskal-Wallis nonparametric one-way analysis of variance (Ghent, 1974; Nie et al., 1975).

## RESULTS

### Clinical observations

Clinical signs were readily apparent in swine given intravascular T-2 toxin. Both the high and low dose animals reacted similarly in the first several hours. They began to chew and salivate within fifteen to thirty minutes. This was followed by persistent vomition and, in some animals, watery diarrhea and flatulence. All the swine were notably restless. Within the first hour most animals exhibited abdominal straining associated with prolonged exhalation, and purplish mucous membranes began to be apparent. Urine output was negligible at two hours after administration of T-2 toxin at the high dose, while swine in the low dose and vehicle groups continued to produce urine. The skin was noticeably reddened, and the ears and limbs were cold to the touch by two to three hours. After three to four hours, the animals appeared drowsy but could be aroused. Animals in the high dose group died as early as five hours after administration of the toxin. The signs of the animals in the low dose group began to regress between five and six hours and at twelve hours, the animals appeared clinically normal except for apparent reductions in alertness and activity. The alterations in body temperature were unusual (Fig. 1). In the high dose group, every animal's temperature either increased or decreased significantly. In the low dose group, there was an early decrease in body temperature between predosing and 1 hour.

## Hemodynamics

The rapid onset of shock induced by T-2 toxin was reflected in an early decline in CO and AOM in both the high and low dose groups (Figs. 2 and 3). In the high dose group, the value of both parameters continued to decline through death. The CO in the low dose group stabilized at about four hours, continued to be stable through eight hours, and by 24 hours was restored to the predosing level. The AOM in the low dose group stabilized between three and four hours. In contrast to the stable nature of CO between four and eight hours, there was an increase in AOM during that time period and thereafter no further increase was detected. At 24 hours AOM was still reduced by 23 to 25 percent of the predosing value.

There was a significant increase in heart rate in both the high and low dose groups. It can be noted in Figure 4; however, that the rate did not increase appreciably until two hours postdosing. No significant trend was detected in the TPR in the high dose group, but the TPR of the low dose group decreased (Fig. 5).

Both high dose and low dose groups exhibited elevations in PVR but for differing reasons (Fig. 6). In the high dose group, PAM increased but then decreased while LAM decreased at each time point (Figs. 7 and 8). In the low dose group, PAM did not increase until 3 to 4 hours, while LAM was decreasing in the zero through four hour period. After four hours, the PAM and LAM of the low dose group increased while CO was stable. After 8 hours, the PVR of the low dose pigs decreased as CO increased, LAM did not change and PAM decreased.

## Plasma catecholamines

Results from the preliminary intravascularly dosed swine revealed that plasma histamine and serotonin concentrations did not increase during T-2

toxicosis, although a shock syndrome and death ensued in all animals. In contrast, the concentrations of norepinephrine and epinephrine were strikingly elevated. In the formal study, tremendous oscillating responses occurred in epinephrine and norepinephrine, in the high dose group (Figs. 9 and 10). There was an elevated but attenuated response in the low dose group. There were no significant changes in the plasma concentrations of dopamine in either the high or the low dose group.

## 6-keto-PGF$_{1\alpha}$ and Thromboxane B$_2$

The concentrations of TXB$_2$ and 6-keto-PGF$_{1\alpha}$ were significantly increased in the T-2 toxin dosed animals (Figs. 11 and 12). The concentration of 6-keto-PGF$_{1\alpha}$ never exceeded the limit of detection in the control group. The plasma concentration of TXB$_2$ rose before 6-keto-PGF$_{1\alpha}$ in both the high and low dose groups.

## Acid-base and blood gas parameters

Although the PaO$_2$ decreased between zero and 2 1/2 hours in both groups, the decline was significant only in the low dose group. Nevertheless the arterial oxygen tension remained functionally adequate in all groups until immediately prior to death (Fig. 13). After 3 hours PaO$_2$ began to rise toward the predosing value and the PaCO$_2$ decreased.

Acidosis became severe early in both groups (Fig. 14). After 2 1/2 hours the acidosis in the low dose group began to resolve. The decline in pH$_a$ in animals that received 4.8 mg/kg T-2 toxin was accompanied by an increase in plasma lactic acid from a concentration of $0.49 \pm .09$ to $7.63 \pm .59$ mmol/L (mean $\pm$ SEM).

## DISCUSSION

There is limited documentation that T-2 toxin has the ability to induce shock. We have investigated numerous parameters in an effort to elucidate the nature of this toxic syndrome. Additional cardiovascular and pathologic determinations were made in these swine and will be reported subsequently.

Between predosing and 4 hours, the rates of decline in CO and AOM in the high dose group were not statistically different from one another. This resulted in the apparent lack of trend in the calculated value of systemic resistance (TPR). However, in other work done in our laboratory in which swine received 4.8 mg/kg T-2 toxin, IV, and were infused with radiolabelled microspheres, virtually no radioactivity was detected in skeletal musculature and decreased perfusion occurred in some visceral organs. These data in addition to our clinical observations of skin and limb perfusion lead to the conclusion that peripheral vascular flow was in fact altered. The calculated resistance value probably reflects the resistance to flow through the limited circuit of the heart, lungs and brain. The absence of any trend in TPR suggests that the closure of the peripheral capillary vasculature was sudden, intense and occurred quite early. In contrast, TPR in the low dose group decreased from predosing through 4 hours.

The rates of decline of CO in the high and low dose groups were not statistically different. Apparently the cardiac effects were similar in both T-2 dosed groups but the peripheral pressor responses resulted in greater reductions in aortic pressure in the low dose group. The microcirculation retained responsiveness in the low dose group as demonstrated during the 4 to 8 hour period. While CO was fairly constant, the vasculature responded to increase systemic pressure and bring the TPR back toward the predosing value.

While these cardiovascular changes were occurring, the concentration of catecholamines was elevated. Inspite of these chronotropic substances, the heart rate did not increase until after a delay. Normally, catecholamines rapidly increase arterial blood pressure, cardiac output, heart rate and total systemic resistance (DeQuattro and Campese, 1981). The action of T-2 toxin apparently did not permit expression of these effects. Thus, many of the effects could be attributable to primary cardiac and vascular factors.

Another hypothesis may also be considered. The early and rapid decline in CO and AOM, the biphasic responses of CO and AOM in the low dose group, the unusual alterations in systemic resistance, the high concentrations of catecholamines reflecting tremendous autonomic activity, the presence of cholinergic symptoms (vomiting, diarrhea, flatulence, salivation) and later, the apparent depression or lethargy of the survivors could be evidence for a significant nervous system component involved in this toxic syndrome. The disturbances in thermoregulation may suggest central mediation.

These effects could also be mediated peripherally. The occurrence of elevated levels of norepinephrine in the presence of decreased blood pressure could support a peripheral mediation theory since the major source of plasma norepinephrine is peripheral sympathetic synapses. It has been debated that elevated concentrations of the α and β agonist, norepinephrine, coincide with hypertension (Lake, 1984; DeQuattro et al., 1984). However, in our experiments blood pressure was consistently depressed. This could suggest an alteration in the response of peripheral vasoactive receptors.

Several years ago it was determined that catecholamines induce platelet aggregates to liberate vasoconstrictor substances (Neil, 1975). Later it was found that thromboxane, in addition to other vasoactive compounds, is produced

by platelets and has the ability to enhance shock by causing pulmonary hyper-
tension, coronary vasospasm and the constriction of bronchial and damaged vas-
cular smooth muscle (Granstrom et al., 1982; Terashita et al., 1978; Frolich
et al., 1980). Moreover, norepinephrine potentiates the in vitro biosynthesis
of thromboxane (Wolfe, 1976). Feedback mechanisms between prostaglandins and
catecholamines have been described that involve the $\alpha$ and $\beta$ receptors, and it
has been proposed that modulation of adrenergic responses may be accomplished
by prostaglandins, including prostacyclin (Ziegler and Lake, 1984; Nasjletti
and Malik, 1982; Stjarne, 1973).

Elevations in the concentrations of 6-keto-$PGF_{1\alpha}$ and thromboxane B2, the
stable hydrolysis products of prostacyclin and thromboxane $A_2$ respectively,
were detected in all T-2 toxin dosed animals and the increase was concurrent
with the rise in catecholamines. This observation is compatible with an
interactions between these substances. Generally, however, the increases in
these humoral substances were delayed as compared to the cardiac and peripher-
al vascular responses, suggesting that the former were secondary responses.

Thromboxane $A_2$, as well as its metabolite, $TXB_2$, have been shown to
induce pulmonary hypertension (Huttemeier et al., 1982; Watkins, et al., 1982;
Friedman et al., 1979; Frolich et al., 1980). In this experiment the rise in
the concentration of $TXB_2$ compares well with the rise in PVR. PVR increased
substantially after 2-3 hours, and remained elevated after 4 hours, whereas
$TXB_2$ concentrations were declining by this time in both groups. In addition
to thromboxane, acidosis and catecholamines may have contributed to the in-
crease in pulmonary resistance (Berk et al., 1977; Webb and Brunswick, 1982).

The early decrease in $PaO_2$ in both groups may indicate that the source of the elevated PVR was primarily arteriolar. The subsequent increase in $PaO_2$ in the low dose group in the face of continued elevation of PVR may indicate a shift to primarily venule constriction. In the presence of severe acidemia, immediate respiratory compensation in the form of hyperventilation and a substantial reduction in $PaCO_2$ would have been expected. However, the fact that $PaCO_2$ of the low dose group decreased, remaining within the range of normal, may indicate an ineffective attempt at respiratory compensation. The absence of appropriate respiratory response may indicate an effect of T-2 toxin on the nervous system. The effect could have been enhanced by the abdominal straining that occurred in these animals and consequent impairment of respiratory exchange.

The concentration of 6-keto-$PGF_{1\alpha}$ increased after $TXB_2$ perhaps as an attempt to balance the effects of thromboxane $A_2$. This increase in 6-keto-$PGF_{1\alpha}$ probably augmented the decrease in systemic blood pressure, since systemic hypotension can be produced by prostacyclin (Collins et al., 1982; Moore et al., 1982).

In summary, there was an early and simultaneous decrease in CO and AOM, and increases in 6-keto-$PGF_{1\alpha}$, catecholamines and heart rate that were accompanied by a decrease in TPR in the low dose group but no change in TPR in the high dose group. An increase in PVR occurred along witn increases in norepinephrine, $TXB_2$ and acidemia.

Finally, there is some evidence for possible nervous system mediation of this toxic syndrome: the sudden decrease in CO and AOM, the differing responses of the microcirculation in the high and low dose groups, the delayed increase in heart rate, the erratic adrenergic response in the high dose group

and the sustained norepinephrine concentrations accompanied by decreased ACH in the low dose group, the presence of cholinergic clinical signs, signs suggestive of mental depression, lack of appropriate respiratory stimulation and altered thermoregulation. F..ther studies must be performed before a theory of nervous system mediation of T-2 toxicosis can be substantiated.

## ACKNOWLEDGEMENTS

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# FOOTNOTES

[1] Supported by the US Army Medical Research and Development Command, Contract No. DAMD 17-83-C-2179

[2] Presented at the FASEB Research Conference, "Diagnosis, toxicity, and therapy of trichothecene mycotoxicosis," June 1984

[3] MycoLab Company, Chesterfield, MO

[4] E. rhusiopathiae bacterin, Dellen Lab, Omaha, NB

[5] Thin layer and gas chromatography, Analytical Toxicology Laboratory, College of Veterinary Medicine, University of Illinois Diagnostic Lab, Urbana, IL

[6] Modulus anesthetic machine with Vernitrol vaporizer, Ohio Med Prod, Madison, WI

[7] Bird Mark 4A ventilator, 3M Corp., Minneapolis, MN.

[8] Tygon tubing, 16 ga., I.D. = 0.05 in., O.D. = 0.09 in., 18 ga., I.D. = 0.04 in., O.D. = 0.07 in., A. Daigger and Co., Chicago, IL

[9] IV infusion pump 2681, Harvard Apparatus Med Prod, Millis, MA

[10] P23ID, Statham Medical Instruments, Gould Inc., Oxnard, CA

[11] Gilson Medical Electronics Inc., Middleton, WI

[12] Cardiogreen, Hynson, Westcott and Dunning Inc., Baltimore, MD

[13] DTL, Gilson Medical Electronics Inc., Middleton, WI

[14] DTCCO-07, Electronics for Medicine, Honeywell, New York, NY

[15] Radiometer BM3MK2, The London Co., Cleveland, OH

[16] Sigma Chemical Co., St. Louis, MO

[17] American Medical Lab, Fairfax, VA

[18] RIA kits, NEK-007A and NEK-008A, New England Nuclear, Boston, VA

[19] Bio-Science Lab, Bellwood, IL

FIGURE LEGENDS

FIG. 1. Rectal temperature of swine given the high (top) and low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal. The alterations in temperature was significant in all animals that received T-2 toxin.

FIG. 2. Alterations in cardiac output (CO) over time in swine given T-2 toxin intravascularly and in swine given the vehicle, only. In the high dose group (o----o), there were only three survivors at seven hours postdosing. (Mean ± SEM).

FIG. 3. Changes in mean aortic blood pressure (AOM) over time in swine given T-2 toxin or vehicle intravascularly. (Mean ± SEM)

FIG. 4. Changes in heart rate (beats per minute) of swine given T-2 toxin intravascularly. (Mean ± SEM)

FIG. 5. Total peripheral resistance (TPR) expressed as a percent of the pre-dosing value in swine given T-2 toxin intravascularly. (Mean ± SEM)

FIG. 6. Pulmonary vascular resistance (PVR) expressed as a percent of the predosing value in swine receiving T-2 toxin intravascularly. (Mean ± SEM)

FIG. 7. Mean pulmonary artery pressure (PAM) in swine receiving T-2 toxin intravascularly. (Mean ± SEM)

FIG. 8. Mean left atrial pressure (LAM) in swine receiving T-2 toxin intra-vascularly. (Mean ± SEM)

FIG. 9. Concentration of plasma epinephrine in swine given the high (top) and low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal.

FIG. 10. Concentration of plasma norepinephrine in swine given the high (top) or low (middle) dose of T-2 toxin and vehicle (bottom) intravascular- ly. Each line represents an individual animal.

FIG. 11. Plasma concentration of thromboxane $B_2$ ($TXB_2$) in swine given the high (top) or low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal.

FIG. 12. Plasma concentration of 6-keto-$PGF_{1\alpha}$ in swine given the high (top) or low (bottom) dose of T-2 toxin intravascularly. Each line repre- sents an individual animal. The concentration of 6-keto-$PGF_{1\alpha}$ never exceeded the limit of detection in the vehicle group.

FIG. 13. Concentrations of arterial blood oxygen ($PaO_2$) and carbon dioxide ($PaCO_2$) in swine given T-2 toxin or vehicle intravascularly. (Mean $\pm$ SEM)

FIG. 14. Alterations of arterial pH ($pH_a$) over time in swine given T-2 toxin or vehicle intravascularly. (Mean $\pm$ SEM)

FIGURE ONE

Body Temperature (°C)

4.8 mg/kg

0.6 mg/kg

Vehicle

Predosing     Hours Post-Dosing



FIGURE TWO

Cardiac Output (ml·min⁻¹·kg⁻¹)

4.8 mg/kg (n = 5)
0.6 mg/kg (n = 5)
vehicle (n = 5)

Predosing     Hours Post-Dosing

FIGURE THREE

Aortic Mean Pressure mmHg vs Hours post-dosing

- 4.8 mg/kg (n = 5)
- 0.6 mg/kg (n = 5)
- vehicle (n = 5)

Predosing



FIGURE FOUR

Heart Rate (Beats/Minute) vs Hours Post-Dosing

- 4.8 mg/kg (n = 5)
- 0.6 mg/kg (n = 5)
- vehicle (n = 5)

Predosing

FIGURE FIVE

4.8 mg/kg (n = 5)
0.6 mg/kg (n = 5)
vehicle (n = 5)



FIGURE SIX

4.8 mg/kg (n = 4)
0.6 mg/kg (n = 5)
vehicle (n = 5)

**FIGURE SEVEN**

Pulmonary Artery Mean Pressure (mmHg) vs Hours Post-Dosing

- 4.8 mg/kg (n = 5)
- 0.6 mg/kg (n = 5)
- vehicle (n = 5)



**FIGURE EIGHT**

Left Atrial Mean Pressure (mmHg) vs Hours Post-Dosing

- 4.8 mg/kg (n = 5)
- 0.6 mg/kg (n = 5)
- vehicle (n = 5)

FIGURE TEN

FIGURE NINE
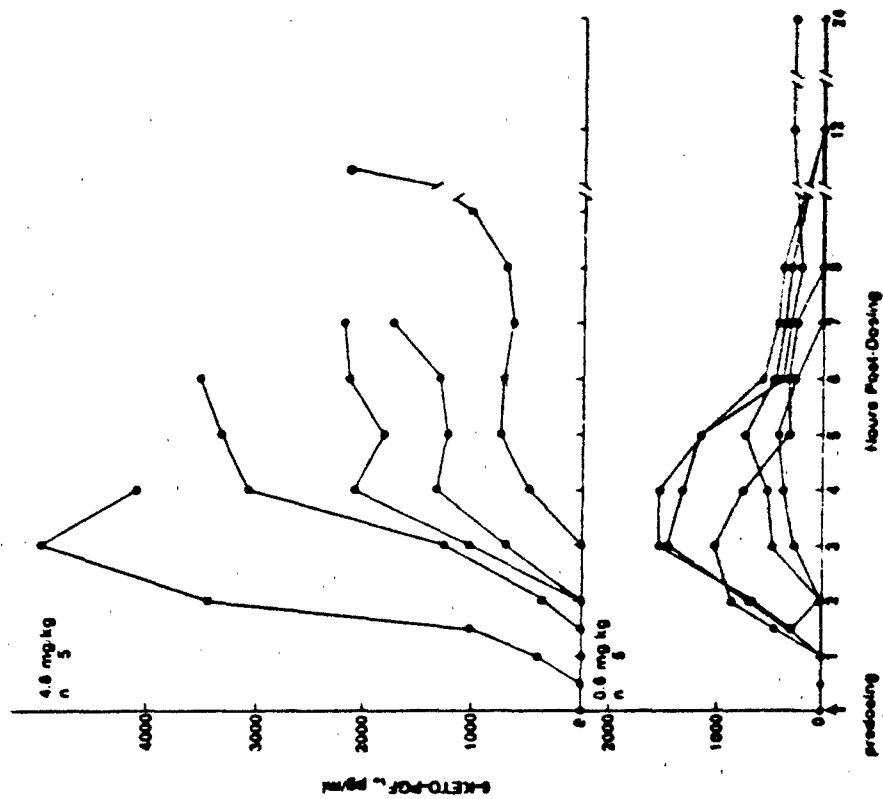
FIGURE TWELVE



FIGURE ELEVEN
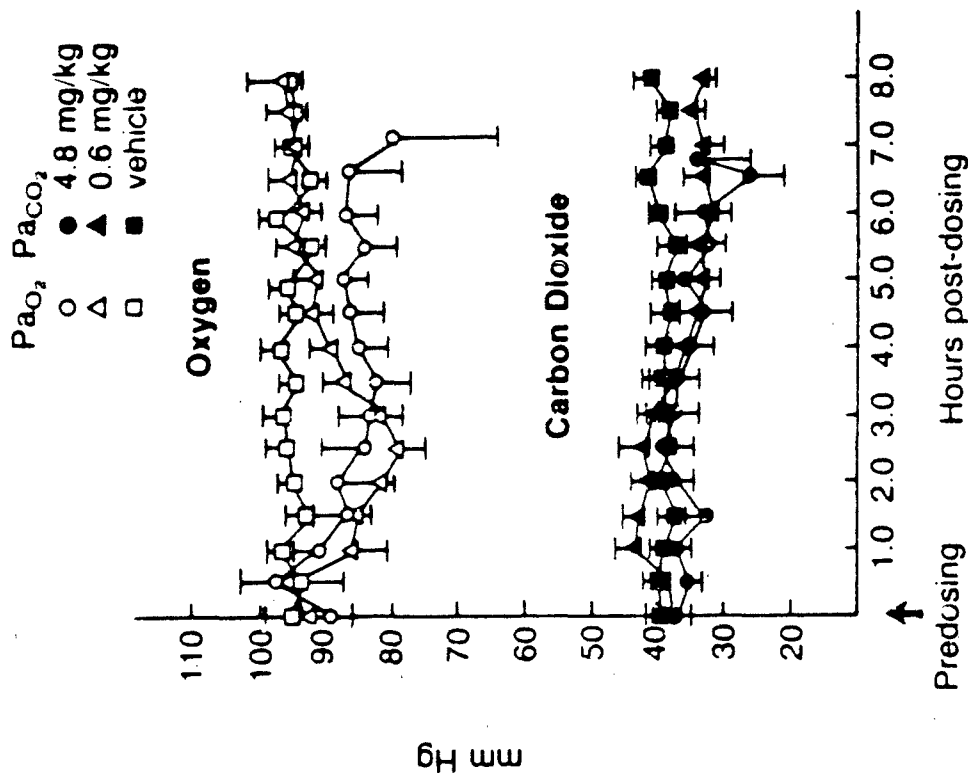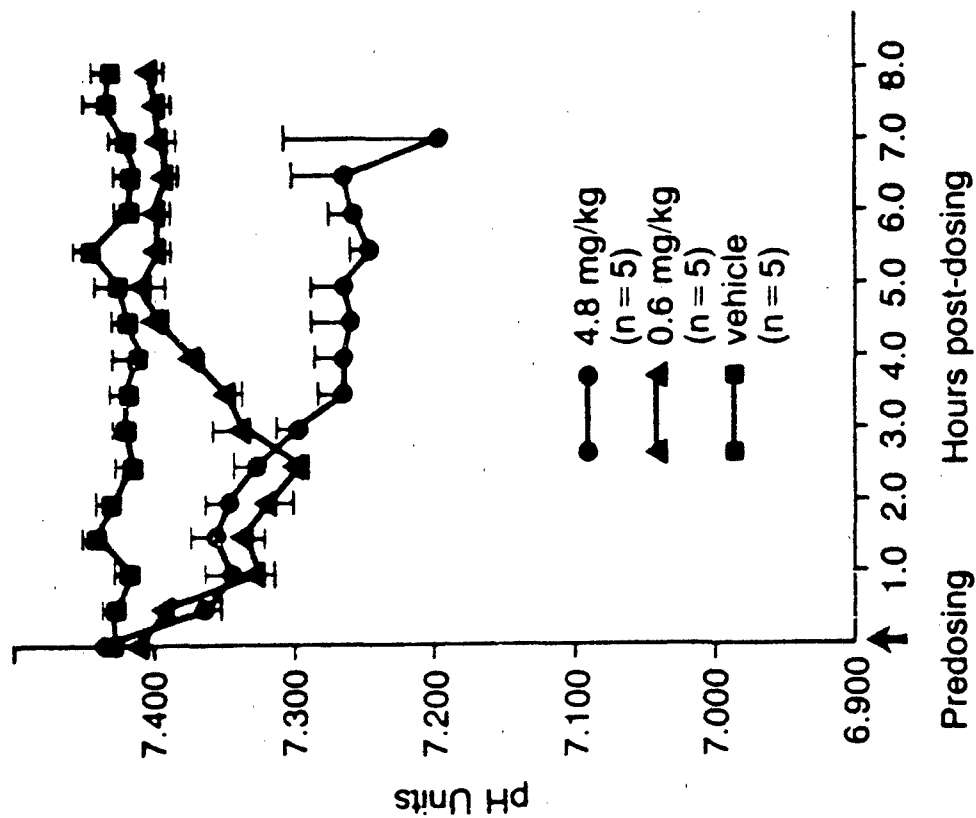
FIGURE FOURTEEN

FIGURE THIRTEEN

APPENDIX B

EXPERIMENTAL T-2 TOXICOSIS IN SWINE.

II. EFFECT OF INTRAVENOUS T-2 TOXIN ON SERUM ENZYMES AND BIOCHEMISTRY,

BLOOD COAGULATION AND HEMATOLOGY.[1,2]

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## ABSTRACT

Experimental T-2 Toxicosis in Swine. II. Effect of Intravenous T-2 Toxin on Serum Enzymes and Biochemistry, Blood Coagulation and Hematology. Lorenzana, R. M., Beasley, V. R., Buck, W. B. (1983). Fundam. Appl. Toxicol. T-2 toxin was given as a single intravascular dose at either 0.6 or 4.8 mg/kg to different groups of fifty kilogram, female swine. Blood samples were taken at hourly intervals for determination of concentrations or activities of the following substances in serum or plasma: creatinine, blood urea nitrogen, inorganic phosphorus, total calcium, ultrafilterable calcium, magnesium, sodium, potassium, chloride, total protein, albumin, cholesterol, glucose, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin. Coagulation analyses included prothrombin time, partial thromboplastin time, activated coagulation time, and fibrin degradation products. Red blood cell, white blood cell, and platelet counts, hemoglobin concentrations and hematocrit, were determined from whole blood samples. An initial leukocytosis was followed by a leukopenia. The numbers of red cells, the hemoglobin concentration, and the hematocrit were increased. Nucleated red blood cells were seen in the blood smears. The serum concentration of bound calcium decreased, while phosphorus, magnesium and potassium increased. Clinical screening tests detected no evidence of a coagulopathy in swine given T-2 toxin intravascularly.

## INTRODUCTION

T-2 toxin, a potent trichothecene mycotoxin produced primarily by members of the genus Fusarium, has been reported to cause infertility (Weaver et al., 1978a), hemorrhagic "moldy corn disease" (Bamburg et al., 1969), vomiting, paresis and death (Weaver et al., 1978b) in swine. In this study, T-2 toxin was given intravascularly to characterize the pathophysiology of the shock syndrome that can be a lethal as well as a sublethal effect in swine (Beasley, 1983; Lorenzana et al., 1984). T-2 toxin has recently been identified as a component of the "Yellow Rain" chemical warfare agent (Rosen and Rosen, 1982). Swine were thus used as a model to study the possible effects of "Yellow Rain" toxicosis in man. In this report, the enzymatic and biochemical changes detected in serum, the effect on blood coagulation and alterations in the hemogram induced by intravenous T-2 toxin are described. Hemodynamic parameters were also determined in these animals and are described in a companion report (Lorenzana et al., 1984).

## MATERIALS AND METHODS

### Chemical and Animals

Purified T-2 toxin[3] was dissolved in 6.5 ml of 70 percent ethanol at room temperature.

White, crossbred, female swine ranging from 40-60 kg were immunized against erysipelas,[4] given an intramuscular injection of selenium and allowed to acclimate to their housing and feed for four to seven days prior to surgical catheterization of the pulmonary artery, aorta, and left atrium. After surgery, the animals were allowed to recover for two to four weeks, and were active and in good health at the time of the study. They were fed a standard ration which contained no detectable concentrations of deoxynivalenol

(vomitoxin), zearalenone, T-2 toxin, diacetoxyscirpenol (DAS) or aflatoxins B1, B2, G1 and G2.[5]

## Surgical Preparation

The manner in which catheters were placed in the pulmonary artery, aorta and left atrium, have been previously described (Lorenzana, et al., 1984).

## Experimental Protocol

Pigs were divided into three groups. A lethal dose of 4.8 mg/kg T-2 toxin which was four-fold higher than the approximate pigs intravascular LD50 (Weaver et al., 1978b) was given to five swine (high dose group), and a sublethal dose of 0.6 mg/kg was administered to five other swine (low dose group). T-2 toxin was given as a single intravascular dose in 6.5 ml of 70 percent ethanol as a vehicle. The six animals in the control group received only the vehicle intravascularly in addition to undergoing identical surgical and handling procedures.

On the day of the study, the conscious animal was restrained in a webbed stanchion which allowed the pig to rest on its sternum and abdomen while its legs hung below the webbing. All swine quickly adapted to this method of restraint and appeared to rest comfortably. After the overlying skin was infiltrated with 2% lidocaine HCl, the catheters were exteriorized through small skin incisions and attached via manifolds to measuring and sampling devices.

Blood samples for baseline (predosing) clinical determinations were collected and the toxin was then administered into the pulmonary artery by a continuous infusion over a five minute period.[6] The beginning of the infusion was considered to be the starting time of the experiment.

Blood samples were collected via the aortic catheter at hourly intervals. All catheters were frequently flushed with heparinized saline to ensure patency and the absence of clots in the samples. Throughout the experiment, each pig received four liters of fluid due to this flushing. The amount of blood taken for clinical determinations was less than ten percent of the animal's total blood volume (i.e.: five hundred milliliters or less). Urine was collected from all experimental animals; however, it was quantitated only in the high dose group.

In the high dose group, samples were taken hourly until the time of death. In the low dose and control groups, samples were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hours after toxin administration. The swine were anesthetized with sodium thiamylal and then killed by exsanguination.

Laboratory Determinations

All blood samples were collected through the aortic catheter. Serum for biochemical and enzymatic evaluations was collected after allowing the blood to clot at 37°C for two hours. The concentrations of creatinine, total protein, albumin, cholesterol, glucose, phosphorus, calcium, blood urea nitrogen, sodium, chloride, potassium, and total bilirubin and the activities of alkaline phosphatase (SAP), alanine amino transferase (ALT) and aspartate aminotransferase (AST) were determined by an autoanalyzer.[7] When the determined values were above the working range of the autoanalyzer, samples were diluted and re-analyzed. Magnesium concentrations were determined by atomic absorption spectrophotometry.[8]

Whole blood was collected in tubes containing EDTA for hematologic evaluation. The hemoglobin concentrations were determined by the cyanmethemoglobin method.[9] Cell and platelet numbers were determined by an electronic

particle counter,[10,11] and the white blood cell differential was determined from blood smears. One hundred cells were observed for every $1 \times 10^4$ white blood cells per ul counted in the total. Plasma prothrombin time and partial thromboplastin time were determined from blood samples to which sodium oxalate had been added.[12] Activated coagulation time was determined by immediately adding freshly collected blood to a premeasured amount of dia- tomaceous earth and determining the number of seconds that transpired from the moment the blood contacted the earth until the first signs of clotting were visible.[13] Serum for quantitation of fibrin degradation products was ob- tained by adding blood to tubes containing thrombin and an enzyme inhibi- tor.[14] All serum and plasma samples were frozen and stored at -20°C prior to analysis. Analyses were performed within five days of collection.

Statistical Evaluation

Widely differing predose values and postdose variances in these data negated standard analyses of variance methods. As an alternative approach, each animal in the three (high, low, and control) groups was tested for sig- nificant upward or downward trends in each parameter, these trends being assessed for significance by both Pearson's r and Kendall's tau two-tailed correlation analyses, with the time sequence of successive observations serving as the independent variate. In this approach, all animals were individually tested against the hypothesis that treatment had no nonrandom effects upon the monitored parameters. Inter-group differences were then judged by comparing the sets of intra-group tests with each other. In this way, for example, five high dose animals, all showing a significant increase in a particular para- meter, are clearly behaving differently than a population of five low dose

animals, all showing either no change or the opposite change in the same para-
meter.

A value of P less than 0.05 was considered statistically significant.
During the early time period when the high dose animals were alive, these
inter-group comparisons were buttressed by Kruskal-Wallis nonparametric one-way
analysis of variance (Ghent, 1974; Nie et al., 1975).

## RESULTS

### Serum Enzymes and Biochemistry

The high dose group had the greatest changes in serum ion concentrations
(Fig. 1). Potassium and magnesium progressively increased while calcium de-
creased. In the low dose group, a decrease in calcium also occurred but the
concentration later began to increase toward the predosing concentration. At
various time points, the concentrations (mg/dl) of ultrafilterable calcium in
the high dose group ranged from $4.0 \pm .2$ to $4.7 \pm .2$, in the low dose group
from $4.5 \pm .4$ to $5.4 \pm .8$ and the control group from $3.3 \pm .5$ to $4.7 \pm .7$
(mean $\pm$ SEM). No trend was observed in the ultrafilterable calcium in any
group during the experiment. It was, therefore, the concentration of bound
calcium that significantly decreased in both high and low dose groups. This
decline in concentration of bound calcium occurred along with a decline in
blood pH (Fig. 2).

In the high dose group, serum concentrations of inorganic phosphorus in-
creased over time (Table 1), whereas there were no trends in the low dose and
vehicle groups. The concentration of blood urea nitrogen became increased in
both the high and low dose groups (Table 1). An increase in serum creatinine
from $1.2 \pm .06$ mg/dl to $2.3 \pm .4$ mg/dl (mean $\pm$ SEM) occurred in the high dose

group only. No significant trends were detected in the low dose and control groups.

The activity of SAP was significantly increased in the high dose group only (Table 1). ALT activity increased significantly from 34.4 ± 3.7 U/l at predosing to 84.3 ± 15 U/l at 7 hours postdosing in the high dose group and from 32.2 ± 3.5 U/l at predosing to 78.6 ± 30.2 at 24 hours postdosing in the low dose group (mean ± SEM). No significant trend occurred in the concentrations of sodium, chloride, total protein, albumin, cholesterol, glucose, and bilirubin or in the activity of AST.

## Hematology

Although the total white blood cell count (WBC) was increased in both high and low dose groups at one hour, thereafter a significant decrease occurred in both of these groups (Fig. 3). In the low dose group, there was a subsequent increase and by 12 hours postdosing the WBC was similar to the predosing number.

The early leukocytosis and the latter leukopenia was due to increases followed by reductions in absolute numbers of both neutrophils and lymphocytes (Figs. 4 & 5). In the low dose group at 24 hours postdosing the number of lymphocytes was approximately 37% of the predosing number. Significant decreasing trends occurred in the absolute numbers of monocytes and eosinophils in the high dose group, but no significant variation occurred in the low dose group. In the high dose group, the number of monocytes per microliter of blood decreased from 631 ± 217 at predosing to zero at 7 hours postdosing (mean ± SEM), and the number of eosinophils per microliter of blood decreased from 837 ± 174 at predosing to 152 ± 55 at 7 hours postdosing (mean ± SEM). No significant trend occurred in any group in the number of bands and basophils.

In the high dose group, between predosing and 7 hours postdosing, significant elevations occurred in the hematocrit, the number of red blood cells and the hemoglobin concentration. The hematocrit (%) increased from 33.4 $\pm$ 2.1 to 38.5 $\pm$ 2.1, the number of red blood cells (1 x $10^6$ per microliter) increased from 7.6 $\pm$ .6 to 9.0 $\pm$ .7 and the concentration of hemoglobin (g/dl) increased from 10.9 $\pm$ .6 to 12.8 $\pm$ .9 (mean $\pm$ SEM). No significant trend in these parameters occurred in the low dose or control groups.

Nucleated red blood cells (metarubricytes) were seen in the peripheral blood smears in both the low and high dose groups (Fig. 6). In the high dose group, the number continued to increase at each successive observation. In the low dose group, their incidence began to decrease after six hours, and by twenty-four hours, no nucleated cells were observed.

## Blood Coagulation Parameters

No significant trends occurred in any of the groups in prothrombin time, partial thromboplastin time, activated coagulation time, platelet number and fibrin degradation products.

## Urine Production

The high dose animals produced an average of 94 ml of urine during the first four hours. No urine was produced by these pigs after four hours. The low dose and control animals produced urine (greater than 300 ml at one urination) and continued to voluntarily micturate throughout the course of the experiment.

## DISCUSSION

There is limited documentation that T-2 toxin has the ability to induce shock. We have investigated numerous parameters in an effort to elucidate the nature of this toxic syndrome. A companion report discusses several of these

parameters. Pathologic (Pang et al., 1983) and blood flow studies conducted in these animals will be reported in future publications.

Alterations in hemodynamics during the shock syndrome resulted in diminished perfusion and contributed to organ dysfunction (Lorenzana et al., 1984). In the high dose group at 4 hours postdosing, when systemic blood pressure fell below an adequate renal filtration pressure of 60 mm Hg, urine production ceased (Guyton, 1976). The serum data would suggest decreased renal clearance of blood urea nitrogen, creatinine, inorganic phoshorus, and potassium. It is likely that the increased serum concentrations of these substances occurred not only as a result of inadequate blood pressure but also from renal vasoconstriction due to elevated circulating catecholamines and thromboxane.

An additional source of the elevated blood urea nitrogen may have been from the breakdown of structural proteins and the increased activity of ALT in both groups may suggest toxic or ischemic damage to visceral or muscular tissues. In the low dose group, systemic pressure was reduced but remained adequate for renal filtration; organ dysfunction was not as severe; and concentrations of cathecholamines and thromboxane were not as great as in the high dose group. Consequently, the rate of increase in the concentration of blood urea nitrogen and ALT in the low dose group was attenuated and there was no significant increase in the concentrations of creatinine, inorganic phosphorus or potassium.

Other circumstances probably contributed to the change in serum ion concentrations in the high dose group. A severe metabolic acidosis occurred in these animals (Lorenzana et al., 1984). The increase in serum potassium could have been augmented by an exchange of intracellular potassium for extracellular hydrogen ions. The increased inorganic phosphorus concentration may have been enhanced by the decrease in serum calcium, blockade of the production of ATP and/or excessive destruction of ATP.

In the high dose group, tissue damage was the most probable source of the increases in serum magnesium and the activity of SAP. In soft tissue, the intracellular concentration of magnesium is greater than the concentration in extracellular fluid. SAP could have escaped fom damaged cells in the liver during the metabolism of T-2 toxin (Beasley, 1983) or from the gastrointestinal tract where necrosis was histologically apparent (Pang et al., 1983).

A probable initiating impetus for the decline in bound calcium was acidic blood pH. Hydrogen ions competitively displace ionic calcium from albumin making it unbound. Homeostatic mechanisms work to maintain a constant concentration of unbound calcium. Since this occurs within minutes, the expected measured effect is a decrease in bound calcium. Subsequent elevations in phosphorus and magnesium may have exerted an additional hypocalcemic effect through hormonal mechanisms (Massry et al., 1970; Gitelman et al., 1968).

Excessive concentrations of plasma epinephrine and norepinephrine, diminished cardiac output and histological evidence of myocardial damage were observed in these animals (Lorenzana et al., 1984; Pang et al., 1983). Adrenergic agents promote myocardial calcium ion influx as a pharmacologic action (Balaza and Bloom, 1982) and an increase in the calcium content of the myocardium has been associated with myocardial lesions (Fleckenstein, 1970; Lehr, 1981). The physiologic alterations induced by direct or indirect action of T-2 toxin may have caused an excessive calcium ion influx sufficient to cause myocardial injury.

Although focal areas of hemorrhage were observed in these swine (Pang et al., 1983), the single intravascular doses of T-2 toxin in this study did not cause hemostatic deficiencies as determined by clinically employed screening tests and numbers of circulating platelets. Other reports have associated T-2

toxin with coagulopathies identified through assessment of individual clotting factors (Gentry and Cooper, 1983; Gentry, 1982).

The pattern of the leukogram of animals that received T-2 toxin reflected a stress response (Schalm et al., 1975). The initial leukocytosis followed by leukopenia resembled an epinephrine response in which cells are shifted from the marginal to the circulating pool of cells. In the low dose group, the neutrophilia, lymphopenia, and eosinopenia at 24 hours postdosing were compatible with the effects of endogenous steroids in response to stress.

The leukopenic period in both high and low dose groups may have been due to sludging in peripheral or pulmonary vessels, margination, destruction, or excessive utilization. Our blood samples were collected from the aorta and, therefore, represent an evaluation of the white blood cell population in major vessels, only. However, during histopathologic examination increased numbers of leukocytes were noted in the liver and lung (Pang et al., 1983).

T-2 toxin is known to have suppressive effects on leukocyte production and to cause lymphoid degeneration in several species (Weaver et al., 1978b; DeNicola et al., 1978; Gentry, 1982; Murphy et al., 1978; Friend et al., 1983; Ueno, 1984). Necrosis and cellular debris in lymphoid tissue and in the bone marrow were observed in these animals (Pang et al., 1983). Lymphocytes have the ability to return to lymphoid tissue after being released into the circulation (Schalm et al., 1975). It is possible that actual lysis of lymphocytes occurred and contributed to the sustained lymphopenia observed in the low dose group.

The appearance of circulating nucleated red blood cells without a concommitant decrease in numbers of mature red blood cells is suggestive of injury to the bone marrow endothelium. Since no nucleated red blood cells were

observed in the low dose group at 24 hours, it can be assumed that the damaging influence, perhaps ischemia or a cytotoxic effect of T-2 toxin or its metabo- lites, was no longer present. Diacetoxyscirpenol and deoxynivalenol, other trichothecene mycotoxins, also cause the appearance of nucleated red blood cells in the peripheral circulation of swine and cattle (Coppock, 1983).

As expected in a multisystem syndrome such as T-2 toxin induced shock, there are combined and complex interactions. In this discussion of numerous biochemical and hematologic parameters, we have examined the effects of T-2 toxin and suggested possible causes; however, further research is needed. In the elucidation of this pathologic state, we have described the result of physiologic alterations and the primary clinical problems; however, the mode of action of the agent must be further characterized in efforts to reach an ultimate goal of control and therapy.

## ACKNOWLEDGEMENTS

## FOOTNOTES

[1] Supported by the US Army Medical Research and Development Command, Contract No. DAMD 17-83-C-2179.

[2] Presented at the Gordon Research Conference on Trichothecene Mycotoxins, June 1983

[3] MycoLab Company, Chesterfield, MO

[4] E. rhusiopathiae bacterin, Dellen Lab, Omaha, NB

[5] Thin layer and gas chromatography, Analytical Toxicology Laboratory, College of Veterinary Medicine, University of Illinois Diagnostic Lab, Urbana, IL

[6] IV infusion pump 2681, Harvard Apparatus Med Prod, Millis, MA

[7] Hycel Super Seventeen Autoanalyzer, Houston, TX

[8] 305A Perkin-Elmer spectrophotometer, Norwalk, CT

[9] Cyanmethemoglobin method, Sigma Chemical Co., St. Louis, MO

[10] Model #ZBI, Coulter Electronics, Hialeah, FL

[11] Camco platelet count, Cambridge Chemical, Ft. Lauderdale, FL

[12] Dade Diagnostic, Inc., Miami, FL

[13] Diatomaceous earth, Becton-Dickinson, Rutherford, NJ

[14] Thrombo-Wellcotest, Wellcome Reagents Limited, England

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## FIGURE LEGENDS

Fig. 1: Serum concentrations of total calcium, potassium and magnesium in swine given T-2 toxin intravascularly. The high dose group demonstrated the greatest alterations in these ions. (mean ± SEM)

Fig. 2: Serum concentration of bound calcium and arterial blood pH in swine receiving 4.8 mg/kg T-2 toxin intravascularly. Both serum calcium and blood pH demonstrated decreases over time. (mean ± SEM)

Fig. 3: Total white blood cell concentrations in blood of swine given T-2 toxin intravascularly. Blood samples were collected from the ascending aorta. An initial leukocytosis was followed by a leukopenia. (mean ± SEM)

Fig. 4: Absolute number of circulating segmented neutrophils in swine given T-2 toxin intravascularly. (mean ± SEM)

Fig. 5: Absolute number of circulating lymphocytes in swine given T-2 toxin intravascularly. (mean ± SEM)

Fig. 6: Nucleated red blood cells in peripheral blood of animals given T-2 toxin intravascularly. No nucleated red blood cells were observed in blood smears of the vehicle dosed animals. (mean ± SEM)

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Total Calcium

4.8 mg/kg (n = 5)
0.6 mg/kg (n = 5)
vehicle (n = 6)

Potassium

Magnesium

Serum Concentration mg/100ml

Predosing    Hours post-dosing

FIGURE TWO

serum calcium
arterial pH

Bound Calcium (mg/dl)

pH Units

Predosing    Hours post-dosing

Legend:
- 4.8 mg/kg (n = 5)
- 0.6 mg/kg (n = 5)
- vehicle only (n = 6)

Total White Blood Cells $1.0 \times 10^3/\mu l$ — Hours post-dosing — Predosing

Legend:
- 4.8 mg/kg (n = 5)
- 0.6 mg/kg (n = 5)
- vehicle (n = 6)

Absolute Number of Segmented Neutrophils $1.0 \times 10^3/\mu l$ — Hours post-dosing — Predosing

FIGURE FIVE

4.8 mg/kg (n = 5)
0.6 mg/kg (n = 5)
vehicle (n = 6)

Absolute Number of Lymphocytes 1.0 x 10³/μl

Predosing    Hours post-dosing



FIGURE SIX

4.8 mg/kg (n = 5)
0.6 mg/kg (n = 5)

Nucleated RBC/μl Blood

Predosing    Hours Post-Dosing

## Table 1
### Serum Biochemical Values In Swine Given T-2 Toxin Intravascularly
Mean (± SEM)

**Hours Post-Dosing**

**Phosphorus (mg/dl)**

| Dose mg/kg | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 12 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4.8 (n=5) | 8.4 (.5) | 7.2 (.2) | 7.5 (.4) | 8.0 (.7) | 10.3 (.1) | 12.3 (2.0) | 11.5 (1.2) | 13.9* (3.0) | | | |
| 0.6 (n=5) | 8.6 (.1) | 6.8 (.2) | 7.1 (.3) | 7.9 (.3) | 8.2 (.3) | 8.0 (.4) | 7.9 (.4) | 8.4 (.6) | 9.3 (.5) | 10.3 (.3) | 8.6 (.5) |
| vehicle (n=6) | 8.2 (.1) | 7.6 (.2) | 8.3 (.3) | 8.1 (.3) | 8.9 (.3) | 8.9 (.4) | 8.8 (.4) | 9.2 (.6) | 8.5 (.5) | 8.8 (.3) | 8.4 (.5) |
| (n) | (3) | (3) | (3) | (2) | (3) | (2) | (1) | (3) | (2) | (3) | (3) |

**Blood Urea Nitrogen (mg/dl)**

| Dose mg/kg | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 12 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4.8 (n=5) | 11.4 (1.2) | 11.4 (1.2) | 13.8 (1.4) | 17.2 (1.7) | 18.8 (3.5) | 24.0 (2.1) | 26.5 (3.0) | 31.0* (3.8) | | | |
| 0.6 (n=5) | 10.0 (1.5) | 10.8 (1.3) | 13.0 (1.3) | 15.6 (1.1) | 17.2 (1.2) | 18.4 (1.2) | 19.4 (1.4) | 21.2 (1.1) | 21.8 (1.7) | 21.2 (2.1) | 10.4 (4.0) |
| vehicle (n=6) | 13.8 (2.7) | 11.5 (1.3) | 11.5 (1.6) | 10.3 (.9) | 9.7 (.3) | 10.1 (1.2) | 9.3 (1.0) | 9.5 (.9) | 8.8 (1.1) | 8.5 (.9) | 11.3 (4.0) |
| (n) | | | | | | | | | | | (3) |

**Alkaline Phosphatase (U/l)**

| Dose mg/kg | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 12 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4.8 (n=5) | 50.4 (5.0) | 62.0 (13.6) | 67.8 (13.4) | 71.8 (11.0) | 102.2 (15.8) | 121.0 (26.4) | 90.8 (7.0) | 110.0* (11.5) | | | |
| 0.6 (n=5) | 55.0 (6.1) | 65.0 (7.8) | 65.4 (7.9) | 61.2 (7.5) | 59.0 (7.4) | 59.2 (8.1) | 60.2 (8.4) | 64.0 (12.1) | 62.3 (11.7) | 67.8 (7.3) | 72.4 (6.9) |
| vehicle (n=6) | 49.5 (5.0) | 48.3 (5.5) | 47.3 (5.1) | 44.3 (5.0) | 45.0 (4.7) | 43.7 (5.0) | 43.1 (4.6) | 43.5 (4.7) | 42.2 (4.3) | 46.5 (5.0) | 49.3 (7.2) |

n   number of individuals per group

*statistically significant trend from predosing through indicated time. $P < 0.05$

APPENDIX C

# TOXICOKINETICS OF THE TRICHOTHECENE
# MYCOTOXIN, T-2 TOXIN, IN SWINE AND CATTLE

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## ABSTRACT

The toxicokinetics of the trichothecene mycotoxin, T-2 toxin, were determined in growing gilts and heifers. Following intravascular administration, the disappearance of the parent T-2 toxin followed a 2-compartment open model with mean elimination phase half-lives of 13.8 and 17.4 minutes and mean apparent specific volumes of distribution of 0.366 and 0.376 1/kg in swine and calves, respectively. The fraction of T-2 toxin eliminated as parent compound in the urine was negligible. In spite of administration of a lethal oral dose in swine (2.4 mg/kg) and a toxic oral dose (3.6 mg/kg) in calves, no parent T-2 toxin was detected in plasma or urine.

After intravascular administration, tissue concentrations of T-2 toxin were consistently highest in lymphoid organs. Tissue residues of T-2 toxin were rapidly depleted such that, in spite of administration of a potentially lethal intravascular dose, no quantifiable T-2 toxin was present in any of the tissues collected at 4 hours after dosing. No T-2 toxin could be detected in liver, even at 1 hour after dosing.

# INTRODUCTION

T-2 toxin is a member of the 12,13-epoxytrichothecene (trichothecene) group of mycotoxins. Field outbreaks of mycotoxicoses in farm animals have been associated with the production of T-2 toxin in feeds in the United States (Hsu et al., 1972), Canada (Puls and Greenway, 1976), Japan (Ueno et al., 1977), and the Soviet Union (Forgacs and Carll, 1962; Joffe and Yagen, 1977). T-2 toxin is produced especially by Fusaria. Isolates of F. sporotrichioides which produced T-2 toxin were consistently isolated from grains associated with alimentary toxic aleukia in humans (Joffe and Yagen, 1977). More recently, T-2 toxin was detected on leaves, in water and in a yellow powder in association with alleged "Yellow Rain" chemical warfare attacks in Asia (Rosen and Rosen, 1983; Mirocha et al., 1983).

With regard to physical and metabolic alterations of T-2 toxin, it was found that T-2 toxin was not altered in significant amounts by human plasma (Ohta et al.) and simulated gastric juice (Ellison and Kotsonis, 1974). Moreover, when F. tricinctum cultures were extracted with water, artificial digestive tract secretions or rat bile for subsequent LD50 studies in rats, all extracts had similar $LD_{50}$'s (Kosuri et al., 1971). When a ruminal fluid extract of the culture was tested, the $LD_{50}$ was almost 2 times as high as any other extract.

The toxicity of T-2 toxin is influenced by mixed function oxidases since inhibition of these enzymes by SKF-525A increased the susceptibility of rats to T-2 toxin, whereas stimulation of enzyme activity by phenobarbital reduced the toxin associated lethality (Kosuri et al., 1971). The administration of radiolabelled T-2 toxin has generally revealed rapid biliary excretion, and significant but temporary concentration of radioactivity in the liver and kidneys (Chi et al., 1978; Matsumoto et al., 1978).

At 18 hours after swine were given radiolabelled T-2 toxin, the voided feces and urine accounted for 25 and 20 percent of the radioactivity respectively (Robison et al., 1979). The concentration of radiolabel in bile was roughly 30 times of that in kidney and liver, the tissues with the highest concentrations. In contrast, spleen contained approximately 1/3 of the renal concentration. Fifty percent of the radioactivity was assumed to be in the gastrointestinal tract.

When a Holstein cow was intubated with T-2 toxin in amounts equivalent to daily consumption of grain containing 50 ppm T-2 toxin for 15 consecutive days and milk was analyzed for the parent compound, concentrations ranged from non-detectable on the 4th and 8th days, to 160 ppb on the 5th day (Robison et al., 1979). Similarly, when a sow was fed a ration containing 12 ppm T-2 toxin, a milk sample contained 76 ppb of the parent compound.

After tritiated T-2 toxin was administered orally to a Jersey cow at 0.42 mg/kg, approximately 70 and 30 percent of the administered radiolabel appeared in the feces and urine, respectively, by 72 hours (Yoshizawa et al., 1981). The authors indicated that, at 4 hours after dosing, unmetabolized T-2 toxin was present at 8 ppb. Only 0.2 percent of the administered radiolabel appeared in the milk. T-2 toxin, HT-2 toxin, neosolaniol, and 4-deacetylneosolaniol accounted for only minor amounts of the radiolabel in urine, milk, and plasma Recently, 3'-hydroxy T-2 toxin and 3'-hydroxy HT-2 toxin were identified as more significant metabolites in specimens of plasma, urine and milk of this cow, but the time course of the occurrence of these metabolites was not thoroughly evaluated (Yoshizawa et al., 1982). At present, the identity and toxicological properties of major metabolites of T-2 toxin in swine have not been described.

The purpose of this study was to develop the ability to predict the rate of disappearance of T-2 toxin from plasma and urine and to determine the likelihood of residues of T-2 toxin in the edible tissues of swine and cattle.

## MATERIALS AND METHODS

### Studies in Swine

Fourteen, 26 to 66 kg (mean 48.8 ± 11.5 SD), female swine of mixed breeding were used. The gilts were immunized against erysipelas,[a] acclimatized for at least 1 week and allotted to 1 of 6 groups. After administration of atropine, anesthesia was induced by inhalation of halothane and oxygen through a mask. An indwelling catheter was surgically implanted in the aorta via the femoral artery. Postoperatively, a foley catheter was inserted into the urinary bladder and the animal was housed in a metabolism cage. The pigs were dosed with T-2 toxin no sooner than 2 days postoperatively. All animals were normal in appearance and behavior at the time of dosing.

The T-2 toxin was solubilized in 2.5 ml of 50 percent ethanol and was administered in singular intravascular doses to 2 gilts in each of groups 1 to 4 at the following doses: 0.0, 0.3, 0.6, and 1.2 mg/kg. Four gilts in group 5 received T-2 toxin intravascularly at 1.2 mg/kg and were euthanized at 1 to 4 hours after dosing. The toxin was administered via the aortal catheter which was subsequently thoroughly flushed with saline and then heparinized saline. Two swine in group 6 were dosed with T-2 toxin intragastrically at 2.4 mg/kg. The dose was solubilized in 2.5 ml of 50 percent ethanol and the tube thoroughly flushed with water. Swine were necropsied immediately after death or euthanasia. Blood samples were collected in heparinized syringes

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[a]  Rhusigen, Pitman-Moore, Inc., Washington Crossing, NJ.

prior to dosing and at postinjection minutes 2.5, 5.0, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 110, 120, 150, 180, 210, 240, 300, 360, 420, 480, 540, 600, and 660. The blood samples were chilled in ice water and centrifuged. The plasma was collected and frozen until assayed. Urine volumes were measured and samples were collected prior to dosing and every 1/2 hour thereafter. Urine samples were kept on ice during collection and then frozen until assayed. Feces and vomitus were collected and put on ice as they became available and subsequently frozen prior to analysis.

Spleen, mesenteric lymph nodes, muscle, kidney, liver, bile, and the contents of the stomach, jejunum, ileum and colon were routinely collected at necropsy and flash-frozen frozen prior to analysis.

Studies in Calves

Seven female calves weighing 201 to 268 kg (mean $238.1 \pm 25.7$ SD) were used in the formal study after being dewormed with thiabendazole, vaccinated against IBR and $PI_3$,[b] and dosed with Leptospira[c] and Clostridial[d] bacterins. After a 3-week or longer acclimation period, the calves were placed in metabolism cages on the day prior to dosing. Bilateral jugular cannulas and foley urinary catheters were inserted on the day of dosing.

In a preliminary study, a calf, C1, was given T-2 toxin intravascularly at 0.15 mg/kg and orally at 0.6 and 1.2 mg/kg. Dosings in this calf were at least 2 weeks apart.

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[b] Nasalgen IP[R], Jensen-Salisbury Laboratories, Division of Burroughs Wellcome Co., Kansas City, MO.
[c] Leptomune S[R], Beecham Laboratories, Bristol, TN.
[d] Clostroid CSN[R], Fort Dodge Laboratories, Fort Dodge, IA.

T-2 toxin in 5 ml of 70 percent ethanol was administered intravascularly to two calves each at 0.6 and 1.2 mg/kg. The orally dosed calves were given crystalline T-2 toxin in dextrose via gelatin capsules. One calf, C2, was given T-2 toxin at 2.4 mg/kg in 2 oral doses spaced 3 weeks apart and was killed at 24 hours after the second dose. Another calf, C3, was given a single oral dose of 3.6 mg/kg. Plasma, urine, and feces were collected as in swine.

One calf, C5, which died after being given T-2 toxin at 1.2 mg/kg, was necropsied immediately after death. Another calf, C7, was given T-2 toxin at 0.6 mg/kg and was euthanized at 6 1/2 hours. The vehicle control calf, C8, as well as C4, dosed at 0.6 mg/kg IV were euthanized at approximately 24 hours. Euthanasia was performed by intravenous injection of pentobarbital followed by exsanguination.

Samples collected from calves at necropsy for T-2 toxin analysis included spleen, mesenteric lymph nodes, muscle, kidney, liver, bile, and the contents of the rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, spiral colon, and large colon. In addition, the brain and heart of calf no. C-5 were analyzed. These samples were flash-frozen prior to analysis.

Analytical Methods

Plasma and Urine. Iso T-2, an isomer of T-2 toxin, was added to samples as an internal standard prior to extraction with benzene. The extracts were partitioned against aqueous sodium hydroxide, chromatographed on a small florisil column, and eluted with chloroform-methanol (95:5 v/v). After concentration of the eluate, the heptafluorobutyryl derivative of T-2 toxin was prepared by reaction with heptafluorobutyrylimidazole (Swanson et al., 1983). The T-2 and iso T-2 derivatives were separated by gas-liquid chromatography at

230° C using a 1.8 meter column containing 3 percent OV-1 and quantitated with a nickel 63 electron capture detector. Recoveries averaged 98 ± 5.5 percent at concentrations ranging from 50 to 1000 ng/ml.

_Tissues_. After addition of the internal standard, iso T-2, the minced tissues were extracted by blending with acetone. Lead acetate was added to the filtrate to precipitate proteins and pigments. The extract was then defatted with hexane. T-2 toxin was partitioned into chloroform, and the chloroform layer was subsequently partitioned against aqueous sodium hydroxide. The chloroform layer was added to a 2.5 g florisil column and the column rinsed with dichloromethane followed by chloroform-acetone (97:3 v/v). T-2 toxin was eluted with chloroform-methanol (95:5 v/v). After concentration of the eluate, the residue was redissolved in 0.5 ml toluene. Derivatization and quantitation was then performed as described for plasma and urine. Recoveries of T-2 toxin in tissues averaged 105 ± 20 percent.

_Bile_. Bile was analyzed by adaptation of a technique developed to quantitate diacetoxyscirpenol in plasma and urine (Swanson et al., 1982). Bile, to which the internal standard iso T-2 was added, was placed on a 500 mg C-18 Bond Elute[e] cartridge. The cartridge was rinsed with water followed by methanol-water (3:7 v/v). T-2 toxin was subsequently eluted with methanol-water (9:1 v/v). Sodium chloride solution was added to the eluate and the mixture was partitioned 3 times with toluene-ethyl acetate (9:1 v/v). Hexane was added to the combined organic layers and transferred to a 1 g florisil column. Thereafter, the method was identical to that used for plasma and urine. Recoveries of T-2 toxin in bile averaged 78 ± 12 percent.

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[e]Analytichem International, Harbor City, CA.

Feces, Vomitus, Gastrointestinal Tract Contents. Except for mincing, these specimens were processed in the same manner as tissues. The mean recovery of T-2 toxin in feces was 77 percent with a standard deviation of $\pm$ 10. In tissues, body fluids, and gastrointestinal contents, the limits of detection and reliable quantitation for T-2 toxin were 25 and 40 ng/ml, respectively.

Data Analysis. Following intravascular administration of T-2 toxin to swine and calves, visual inspection of semilogarithmic plots of plasma concentrations vs. time suggested a biexponential equation of the form $Cp = Ae^{-\alpha t} + Be^{-\beta t}$ where Cp is the plasma concentration at a given time t, A and B are coefficients and $\alpha$ and $\beta$ are exponents. Initial estimates of the parameters of the equation were derived by the "method of residuals" (Gibaldi and Perrier, 1982). Using these estimates, best fit values were determined using a digital computer (IBM 360/75) and an iterative least squares fitting program, the SAAM-27 program (Berman and Weiss, 1968). The parameters of the biexponential equation were then solved for the first order rate constants of a two compartment open model (Fig. 1). Inspection of the plasma data of P17 and C5 revealed that, at the high dose, these were the individuals of each species with the slowest elimination phases as well as the most pronounced transition between $\alpha$ and $\beta$ phases. To determine whether a more complex model would better explain the disappearance of T-2 toxin from plasma, the data of P17 and C5 were fitted to a 3 compartment open model. An F test (Boxenbaum et al., 1974) of the weighted sums of squared deviations of the data points from the predicted points of the two and three compartment models was used to determine whether significant improvement of fit had resulted from the use of the more complex model.

The terminal portion of the plasma curve of P21 was skewed upward due to comparatively similar values of 53, 55, and 48 ng/ml at 55, 60 and 70 minutes, respectively. The plasma T-2 concentration at the next time point, 80 minutes, had declined to the limit of detection. It was apparent, therefore, that the least squares computation had entered a local minimum in the fitting of estimates of the parameter values (Boxenbaum et al., 1974) which caused inappropriate estimation of the terminal slope. Therefore, in order to obtain best fit estimates of the kinetic parameters for P21, the program was run with ß fixed at the mean ß value of the other 5 animals at this dose.

In order to assess whether dosage had influenced the toxicokinetics of T-2 toxin, the best fit values derived at each of the two doses in calves were compared by t-tests (Steel and Torrie, 1960). Similarly, the kinetic values obtained over the three doses in swine were compared by a one-way analysis of variance as well as by linear and curvilinear regression using a computer program.[f]

## RESULTS

Individual and mean pharmacokinetic parameters determined for intravascularly dosed swine and cattle are shown in Tables 1 and 2. Figures 2 and 3 are curves calculated by the means of the kinetic parameters at each dosage to illustrate the agreement with the mean T-2 concentration at each time point. In both P17 and C5, no significant improvement in the sum of squares resulted from the use of the 3 compartment model, as compared to the two compartment model.

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[f]  Statistical Package for the Social Sciences, SPSS, Inc., Chicago, IL.

One-way analysis of variance of the effect of different intravascular dosages of T-2 toxin on the toxicokinetic parameters of swine did not reveal any significant differences between the kinetic parameters of the three groups. The calculation of linear regression coefficients similarly revealed no linear trend in any of the parameters. When analyzed by multiple regression, a curvilinear trend was apparent in only one parameter, total body clearance, which declined at an increasing rate as dosage increased (Table 3 and Fig. 4). In calves, t-tests of the toxicokinetic parameters revealed no differences between the two intravascular dosages. Also in calves, the mean total body clearances in the 0.6 and 1.2 mg/kg intravascular groups were essentially the same.

T-2 toxin rapidly disappeared from the plasma of intravascularly dosed swine and cattle. By two hours after intravascular administration of T-2 toxin at 1.2 mg/kg, the toxin could no longer be detected in the plasma of either species. Although renal shutdown occurred in individuals of both species at the 1.2 mg/kg intravascular dose, urine production continued at lower doses. Regardless of which species and which dosage of T-2 toxin was employed, urinary excretion of T-2 toxin was inconsistent, and less than 0.1 percent of the dose was recovered in urine as parent compound.

Analysis of tissues of swine dosed with T-2 toxin intravascularly at 1.2 mg/kg and euthanized after 1 to 4 hours revealed rapid disappearance of the parent compound (Fig. 5). The lymphoid organs, spleen and mesenteric lymph nodes, contained the highest concentration of parent T-2 toxin. Muscle tissue contained more of the parent compound than kidney, in which T-2 concentrations were below the limit of reliable quantitation even at one hour. No T-2 toxin

was detected in liver or adipose tissue at any time. The analysis of bile revealed the presence of T-2 toxin in only one animal and then the toxin was present only at concentrations below the 40 ppb limit of reliable quantitation. This pig, P17, had received intravascular T-2 toxin at 1.2 mg/kg and was euthanized at 130 minutes. Calves and other swine, including P15 which was euthanized at 1 hour after an intravascular dose of 1.2 mg/kg, did not have detectable T-2 toxin in bile.

After P17, P21 and P26 received T-2 toxin intravascularly at 1.2 mg/kg, no parent compound was detected in 15 specimens of vomitus collected from 15 to 120 minutes after dosing. However, after intragastric administration of T-2 toxin at 2.4 mg/kg, vomitus accounted for approximately 12 and 36 percent of the dose in P14 and P16, respectively.

Swine nos. P15, P26, and P13 were dosed intravascularly at 1.2 mg/kg and were necropsied at 1.1, 3, and 12.3 hours after dosing, respectively. T-2 toxin was present, but not at quantifiable concentrations, in the stomach contents of P15. However, no T-2 toxin was detected in jejunum, cecum, and colon contents of this animal nor was the parent compound detected in any of the gastrointestinal tract contents of P26 and P13. Similarly, feces from swine dosed intravascularly with T-2 toxin at 0.6 or 1.2 mg/kg and voided at 2, 20, 25, 30, 118, 202, 209, 210, and 345 minutes postdosing contained no detectable T-2 toxin.

No T-2 toxin was detected in 4 specimens of feces voided by P14 from 3.2 to 8.5 hours after intragastric dosing at 2.4 mg/kg. However, the stomach, jejunal, and spiral colon contents of this pig, which died at 19.5 hours after dosing, contained 16.4 ppm, 374 ppb, and 66 ppb of T-2 toxin, respectively.

The ileum and cecum of this animal were essentially empty. These results were somewhat different than those of P16, which died 18 hours after the same intragastric dose. The stomach contents of P16 contained 3.3 ppm while the contents of the ileum, jejunum, cecum, and colon were all free of detectable concentrations of T-2 toxin.

No T-2 toxin was detected in any tissues from cattle dosed orally or intravascularly with T-2 toxin. These included: C2 dosed with 2.4 mg/kg orally and euthanized at 24 hours; C7 dosed with 0.6 mg/kg intravenously and euthanized at 5 1/2 hours; C5 which was dosed at 1.2 mg/kg and died at 10 1/2 hours; and calves euthanized at approximately 24 hours after intravascular doses of 0.6 or 1.2 mg/kg. After C3 was dosed orally with T-2 toxin at 3.6 mg/kg, no intact T-2 toxin was detected in feces collected at 90, 170, 195, 585, 785, 1875, 2910, 3510, and 4260 minutes after dosing. Concentrations of T-2 toxin which were detected in the gastrointestinal tract of C2, at 24 hours after an oral dose of 2.4 mg/kg, included 62 and 40 ppb in rumen and omasum contents, respectively. No T-2 toxin was detected in contents of the reticulum, abomasum, jejunum, cecum, or spiral colon, or in the feces of this animal.

## DISCUSSION

It could not be concluded that the toxicokinetics of T-2 toxin in swine and calves were independent of dosage for two reasons. First, the number of animals at most dosages was small, and second, there was a decrease in total body clearance with increasing dosage in swine. However, it was concluded that the differences within these dosages were small, inasmuch as the range of values for mean total body clearance values in swine was from 57 to 92 ml/kg/min. Because of the rapid disappearance of the parent compound, the terminal portion

of the plasma concentration curve was less clearly defined at the lower dosages and this could have lead to an over-estimation of the total body clearance. In P4 and P9, plasma concentrations fell below the limit of detection at 20 and 25 minutes, respectively.

It has been reported that, in swine, the fractions of total cardiac output distributed to the liver (via the hepatic artery), and to the gastrointestinal tract were 4.27 and 18.71 percent, respectively (Tranquilli et al., 1982). These values, based on a cardiac index value of 135 ml/min/kg (Tranquilli et al., 1982), are equivalent to approximately 5.8 and 25.7 ml/kg/min or an approximate sum of 31.5 ml/kg/min. Therefore, even if one were to assume: that the gastrointestinal and hepatic blood flow was normal during T-2 toxicosis; that 100 percent of the gastrointestinal blood flow was delivered via the portal vein to the liver; that 100 percent of the T-2 toxin in plasma in the portal and hepatic arteries was cleared; and that an equilibrium occurred such that plasma concentrations of T-2 toxin remained proportional to the overall concentration of parent compound in the body as a whole; then the amount delivered to the liver would be insufficient to account for the clearance of T-2 toxin in this species. It is possible, therefore, that blood flow was altered or that metabolism in tissues other than the liver and gastrointestinal tract also contributed to the rapid disappearance of T-2 toxin from plasma.

The failure to find any detectable T-2 toxin in liver, even at 1 hour after intravascular dosing, is nevertheless compatible with the suggestion that this organ is a primary site of T-2 toxin modification. Although it appears that the liver and gastrointestinal tract may not be the only tissues contributing to the disappearance of T-2 toxin after intravascular dosing, the

absence of T-2 toxin in liver after intravascular dosing, in conjunction with the failure to detect T-2 toxin in plasma or urine at any time after intragastric or oral administration, indicates a very active first-pass effect.

The minimal excretion of T-2 toxin in swine and bovine urine contrasts with the urinary recovery of 30 percent of the radiolabel from orally-administered, tritiated T-2 toxin in a previous study (Yoshizawa et al., 1981). These authors also reported that 44 and 11 ng/ml of T-2 toxin were present in urine as parent compound at 12 and 24 hours after dosing respectively. The present study would suggest that even less T-2 toxin is eliminated as parent compound by this route.

The results of the present study indicate that, in swine, T-2 toxin is relatively poorly absorbed from and is stable in the stomach; the latter finding is in agreement with the in vitro studies of Ellison and Kotsonis, 1974. With regard to gastric absorption, it must be noted that swine given T-2 toxin intragastrically entered into a state of shock terminating in death within 20 hours. The alterations in circulation from shock and the local effects of T-2 toxin undoubtedly affected the absorptive and metabolic functions of the gastrointestinal tract. Nevertheless, after T-2 toxin entered the small intestine, absorption and/or metabolic alteration rapidly occurred. Although it was previously demonstrated (Robison et al., 1979; Chi et al., 1978; Matsumoto et al., 1978) that radiolabel from T-2 toxin was eliminated via the bile, intact, unconjugated T-2 toxin was not eliminated in significant amounts in the bile of these calves or swine.

In calves, ruminal dilution would undoubtedly delay absorption. Calves administered T-2 toxin orally survived the shock syndrome and were recovering at 24 hours. It is therefore likely that, in calves, the gastrointestinal

tract blood supply and function were somewhat less affected. The possibility of binding to, or inactivation by, components of the rumen fluid must also be considered in view of the evidence suggesting that rumen fluid may lessen the toxicity of Fusarium extracts (Kosuri et al., 1971).

Another consideration is that the swine received T-2 toxin in an ethanol-water (50:50 v/v) solution which was flushed into the stomach with water. Although the water probably precipitated some of the T-2 toxin in swine, calves received 100 percent of the dose in crystalline form. Therefore, in addition to the obvious differences between the rumen contents of calves and the stomach contents of swine, dissolution of T-2 toxin must also be considered. Nevertheless, in view of the pronounced clinical signs of T-2 toxicosis in C2 and C3; the failure to recover T-2 toxin in the feces of these calves; and the minimal amounts of T-2 toxin in the gastrointestinal tract contents of C2; dissolution and absorption undoubtedly occurred over the course of the experiment.

After intravascular administration, lymphoid tissues consistently had the highest (as high as 162 ppb) T-2 toxin concentrations. Although lymphocytes are most sensitive to T-2 toxin, it cannot be concluded that the presence of higher concentrations of the parent compound was a primary result of binding to these tissues. Previous distribution studies with T-2 toxin in swine revealed much higher radiolabel concentrations in liver and kidney than in spleen (Robison et al., 1979). It is probable, therefore, that less of the T-2 toxin which reached the lymphoid tissues was metabolized. This may have occurred as a result of cytotoxic effects in these T-2 sensitive cells and possibly may have been aggravated by a secondary reduction in blood supply to these damaged tissues.

It is concluded that significant residues of T-2 toxin as parent compound are unlikely to be encountered in the edible tissues, urine, feces, or intestinal contents of swine and cattle, nor is it likely that they would be found in milk. In view of the severe toxicosis in swine and cattle dosed orally in this study, in which the T-2 toxin was never present at detectable concentrations in plasma; since the disappearance of T-2 toxin is accompanied by the appearance of toxic metabolites, such as the recently identified principal metabolites in bovine plasma, urine and milk; and since these metabolites are approximately as toxic as the parent compound (Yoshizawa et al., 1981), the absence of residues of the parent compound does not amount to detoxification.

Due to dilution of the toxin in the animal and the absence of accumulation of T-2 toxin or metabolites in animal tissues, the risk to the human consumer of animal products containing T-2 metabolites is definitely less than the risk to the animal consuming T-2 toxin. However, until an adequate data base with regard to the subacute and chronic effects of T-2 toxin and metabolites on clotting, hematopoietic and immune functions is established, withdrawal from heavily contaminated feed prior to slaughter would be appropriate. Fortunately, by 72 hours after administration of labelled T-2 toxin, tissue:feed ratios of T-2 equivalents in the cow ranged from 0.0003 to 0.0006 (Yoshizawa et al., 1981). Therefore, after three days on toxin-free feed, the concentrations of metabolites of T-2 toxin likely to be encountered in animal products are unlikely to be of toxicologic consequence.

With regard to tissues and body fluids, efforts to identify and characterize the time course of principal metabolites of T-2 toxin are of greater diagnostic benefit than analysis for the parent T-2 toxin. In instances where feedstuffs are unavailable for analysis, it would be reasonable to analyze

stomach or rumen contents for T-2 toxin, provided that suspect feed was still being consumed antemortem. Diagnosticians should be aware of the possible dermal, oral, gastrointestinal, reproductive, immunosuppressive, and possibly hemorrhagic effects of the more toxic trichothecene mycotoxins in addition to their preponderance to cause feed refusal.

In this study, it was clear that the development of clinical signs of weakness, circulatory shock, somnolence, and gastrointestinal disturbance as well as lesions in the lymphoid tissues and gastrointestinal tract was progressing after essentially complete disappearance of the parent compound. It is arguable, therefore, that the metabolites of this toxin probably cause significant if, not the major effects of T-2 toxicosis. Work in progess in our laboratories has revealed that 3'OH HT-2 toxin and glucuronide conjugates of 3'-OH HT-2, 3'-OH T-2, HT-2 and T-2 are the primary metabolites in swine. The time course of occurrence of these compounds will be characterized in subsequent reports.

## ACKNOWLEDGEMENT

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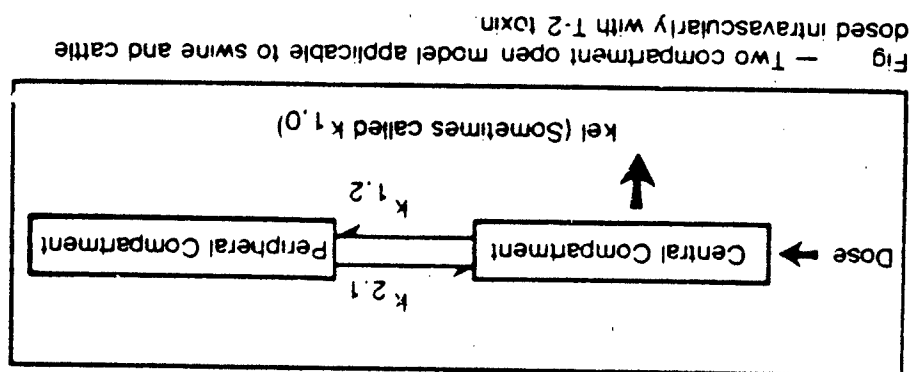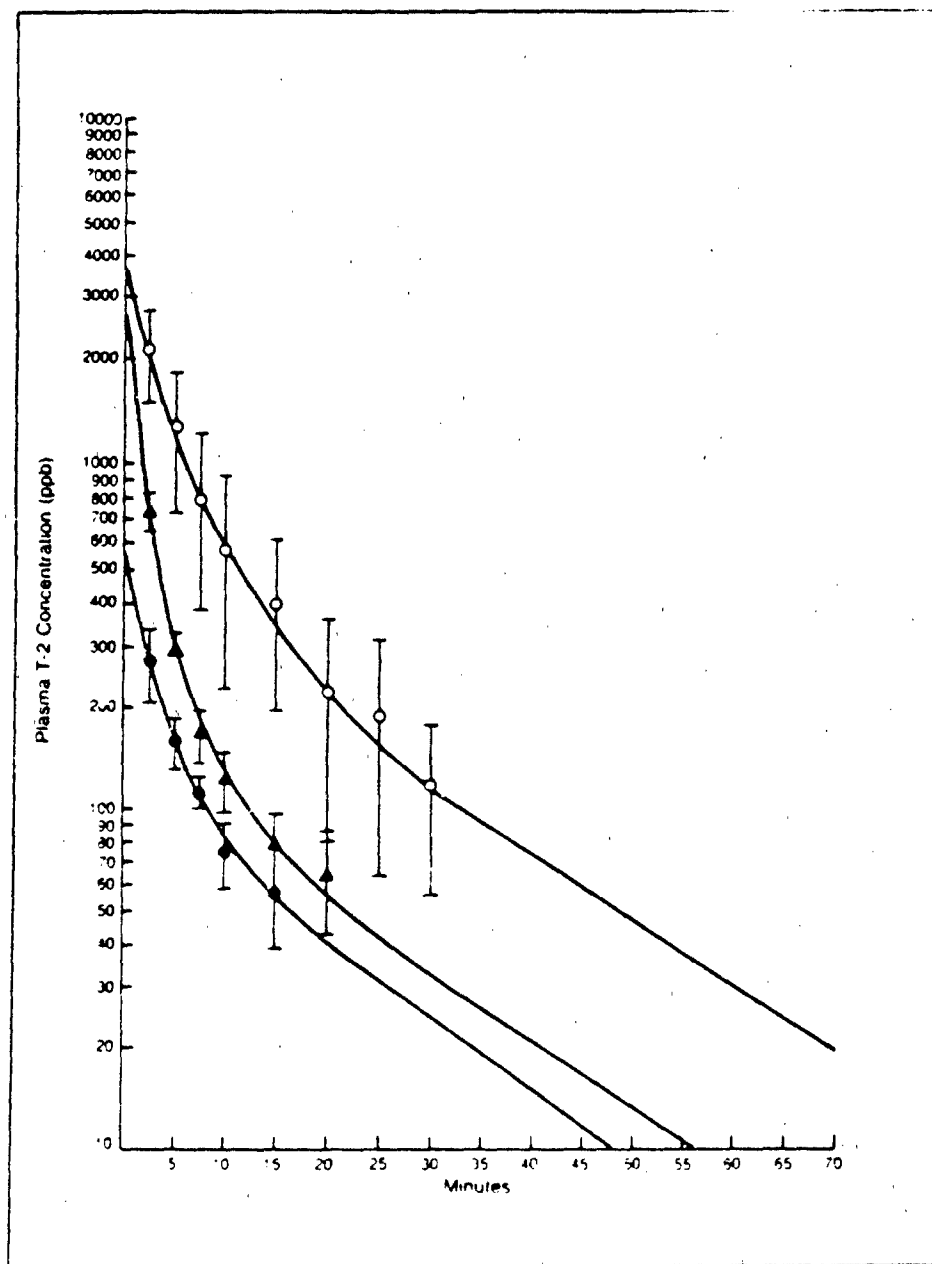
Fig. 2. Mean $\pm$ SD of swine plasma T-2 concentrations after 1 2 ( O ), 0.6 ( ▲ ), or 0.3 ( ● ) mg/kg, intravascularly.
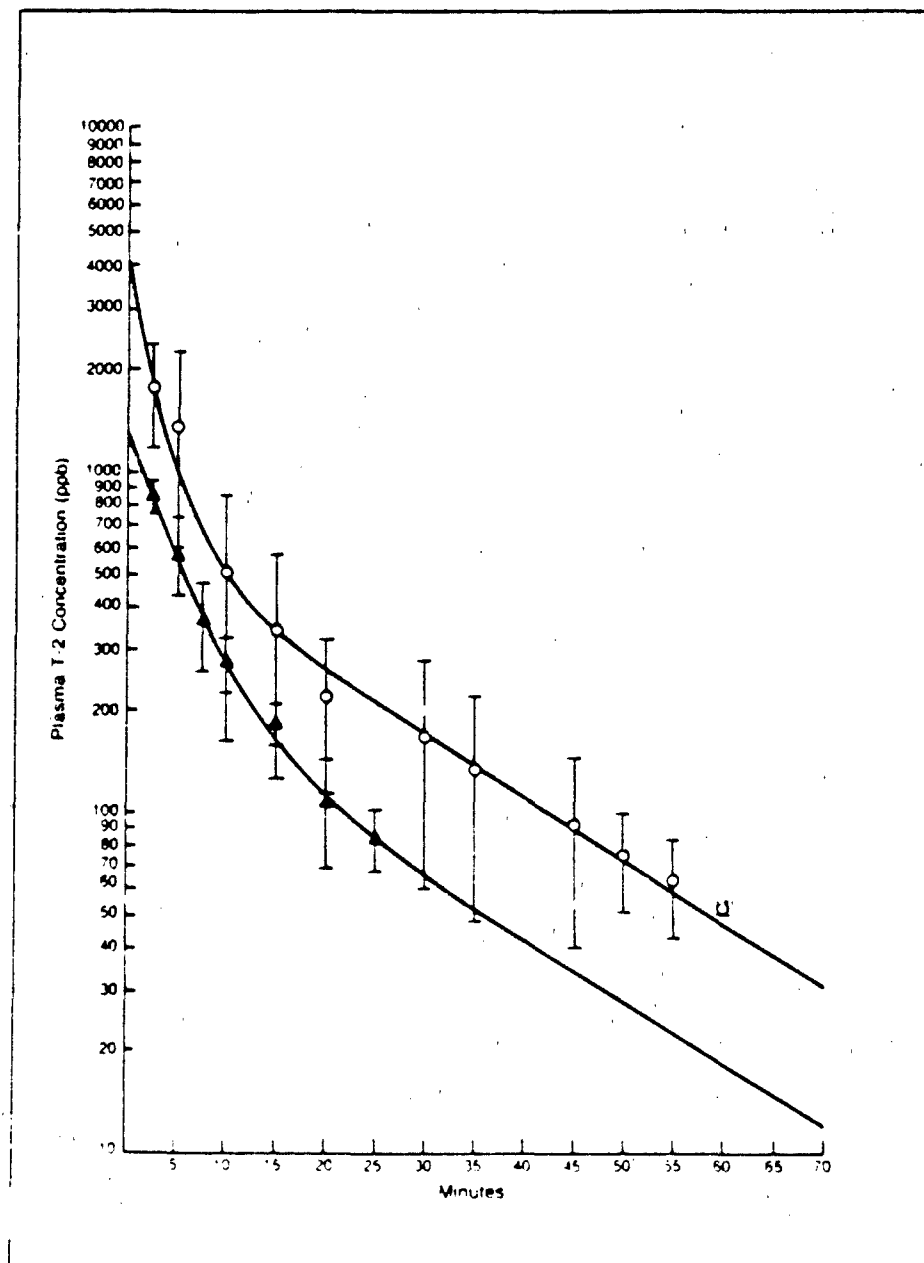
Fig. 3. Mean ± SD of bovine plasma T-2 concentrations after 1.2 (O) or 0.6 (▲), IV.
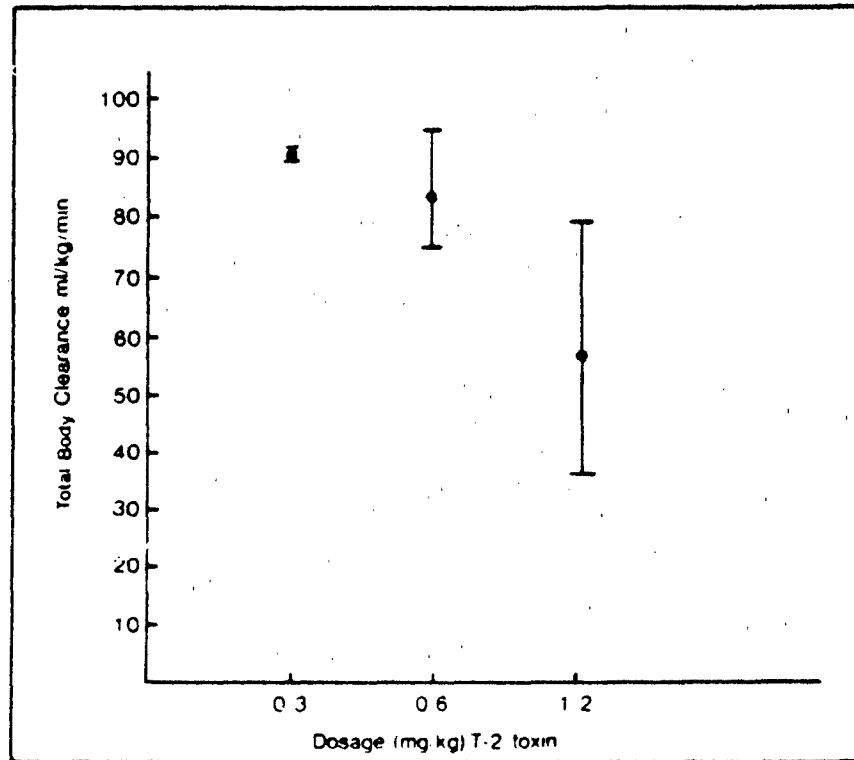
Fig     — Total body clearance of T-2 toxin in swine following intravascular administration

Fig. 4.   Total body clearance of T-2 toxin in swine (mean $\pm$ SD) as a function of intravascular dosage.

Fig. 5. Swine T-2 toxin concentrations in tissues when euthanized from 1 to 4 hours after intravascular doses of 1.2 mg/kg. T-2 toxin was never present at detectable concentrations in the liver.

## TABLE 1.

Estimates of Toxicokinetic Parameters in Swine After Intravascular Dosing with T-2 Toxin

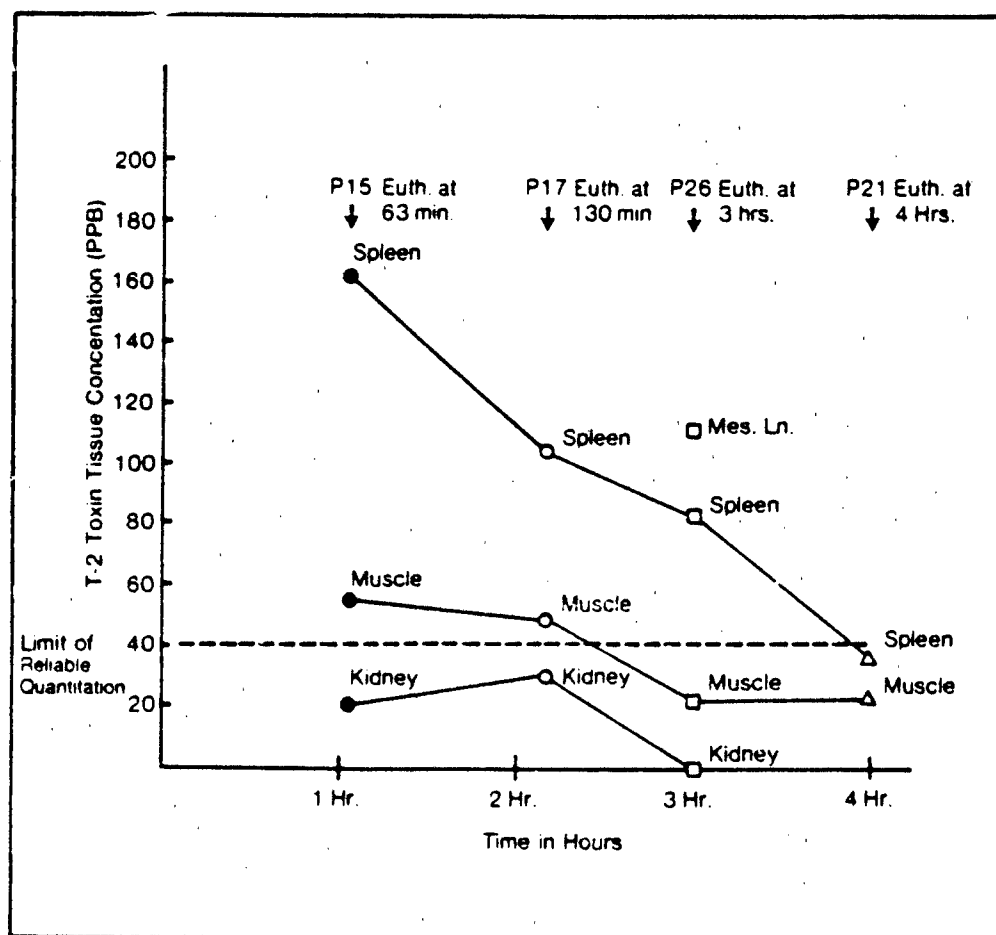| Animal Number | Dose (mg/kg) | Alpha $(min^{-1})$ | Beta $(min^{-1})$ | A (ng/ml) | B (ng/ml) | A+B (CPo) (ng/ml) | Kel $(min^{-1})$ (K-1,0) | Vdc (1/kg) | T-1/2 beta (min) | T-1/2 alpha (min) | K-1,2 $(min^{-1})$ | K-2,1 $(min^{-1})$ | T'CL ml/kg/min |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P4 | 0.3 | 0.371 | 0.058 | 613 | 92 | 705 | 0.218 | 0.426 | 11.95 | 1.87 | 0.133 | 0.099 | 93 |
| P9 | 0.3 | 0.500 | 0.061 | 283 | 165 | 448 | 0.137 | 0.670 | 11.36 | 1.39 | 0.201 | 0.223 | 92 |
| x̄ | | 0.436 | 0.060 | 448 | 129 | 577 | 0.178 | 0.548 | 11.66 | 1.63 | 0.167 | 0.161 | 92.5 |
| ±SD | | ±0.091 | ±0.002 | ±233 | ±52 | ±182 | ±0.057 | ±0.173 | | | ±0.048 | ±0.088 | ±0.7 |
| | | | | | | | | | | | | | |
| P7 | 0.6 | 0.576 | 0.046 | 2748 | 166 | 2914 | 0.348 | 0.206 | 15.07 | 1.20 | 0.213 | 0.076 | 72 |
| P12 | 0.6 | 0.579 | 0.101 | 1608 | 349 | 1957 | 0.314 | 0.307 | 6.86 | 1.20 | 0.180 | 0.186 | 96 |
| x̄ | | 0.578 | 0.074 | 2178 | 258 | 2436 | 0.331 | 0.257 | 10.97 | 1.20 | 0.197 | 0.131 | 84.0 |
| ±SD | | ±0.002 | ±0.039 | ±806 | ±129 | ±677 | ±0.024 | ±0.071 | | | ±0.023 | ±0.078 | ±17.0 |
| | | | | | | | | | | | | | |
| P8 | 1.2 | 0.488 | 0.063 | 4018 | 1071 | 5089 | 0.202 | 0.236 | 11.00 | 1.42 | 0.197 | 0.152 | 48 |
| P13 | 1.2 | 0.344 | 0.069 | 3876 | 1676 | 5552 | 0.156 | 0.216 | 10.04 | 2.01 | 0.105 | 0.152 | 34 |
| P15 | 1.2 | 0.242 | 0.033 | 3388 | 268 | 3596 | 0.162 | 0.334 | 21.00 | 2.86 | 0.063 | 0.049 | 54 |
| P17 | 1.2 | 0.240 | 0.026 | 1855 | 204 | 2059 | 0.132 | 0.583 | 26.65 | 2.89 | 0.087 | 0.047 | 77 |
| P21 | 1.2 | 0.163 | 0.052 | 1957 | 895 | 2852 | 0.098 | 0.421 | 13.33 | 4.25 | 0.031 | 0.087 | 41 |
| P26 | 1.2 | 0.478 | 0.067 | 4248 | 315 | 4563 | 0.336 | 0.263 | 10.34 | 1.45 | 0.114 | 0.095 | 88 |
| x̄ | | 0.326 | 0.052 | 3324 | 738 | 3952 | 0.181 | 0.342 | 15.39 | 2.48 | 0.100 | 0.097 | 57.2 |
| ±SD | | ±0.135 | ±0.018 | ±1059 | ±583 | ±1352 | ±0.083 | ±0.140 | | | ±0.056 | ±0.047 | ±21.1 |

## TABLE 2.

Estimates of Toxicokinetic Parameters in Calves After Intravenous Dosing with T-2 Toxin

| Animal Number | Dose (mg/kg) | Alpha (min$^{-1}$) | Beta (min$^{-1}$) | A (ng/ml) | B (ng/ml) | A+B (CPo) (ng/ml) | Kel (min$^{-1}$) (K-1,0) | Vdc (l/kg) | T-1/2 beta (min) | T-1/2 alpha (min) | K-1,2 (min$^{-1}$) | K-2,1 (min$^{-1}$) | T'CL (ml/kg/min) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C4 | 0.6 | 0.202 | 0.033 | 1043 | 139 | 1182 | 0.126 | 0.508 | 21.00 | 3.43 | 0.054 | 0.053 | 64 |
| C7 | 0.6 | 0.208 | 0.047 | 1146 | 295 | 1441 | 0.123 | 0.416 | 14.74 | 3.33 | 0.050 | 0.080 | 51 |
| x | | 0.205 | 0.040 | 1095 | 217 | 1312 | 0.125 | 0.462 | 17.87 | 3.38 | 0.054 | 0.067 | 57.5 |
| ± SD | | ±0.004 | ±0.010 | ±72 | ±110 | ±183 | ±0.002 | ±0.065 | | | ±0.001 | ±0.019 | ±9.2 |
| C5 | 1.2 | 0.442 | 0.039 | 4020 | 309 | 4329 | 0.254 | 0.277 | 17.77 | 1.57 | 0.159 | 0.068 | 70 |
| C9 | 1.2 | 0.252 | 0.043 | 3199 | 789 | 3988 | 0.128 | 0.301 | 16.12 | 2.75 | 0.082 | 0.084 | 39 |
| x | | 0.347 | 0.041 | 3610 | 549 | 4159 | 0.191 | 0.289 | 16.95 | 2.16 | 0.121 | 0.076 | 54.5 |
| ± SD | | ±0.134 | ±0.003 | ±581 | ±339 | ±241 | ±0.089 | ±0.017 | | | ±0.054 | ±0.011 | ±21.9 |

* Legend for Tables 1 and 2--Alpha and Beta were derived from the slopes of the distributive and elimination phases which are $-\alpha/2.303$ and $-\beta/2.303$, respectively. A and B are the respective intercepts of the distributive and elimination phases. A + B is the theoretical plasma concentration at time zero. Kel represents the rate constant for the disappearance of T-2 toxin from the central compartment. Vdc is the volume of distribution of the central compartment. K-1,2 and K-2,1 are rate constants for the processes shown in Figure 3. T'Cl is the theoretical rate of total body clearance and is calculated from $(K_{el}) \times (Vd_c)$.

## TABLE 3.

Analysis of Variance Table for Multiple Regression Analysis
of the Effect of Dosage on Total Body Clearance
of T-2 Toxin in Swine.

| Source | D.F. | Sum Squares | Mean Squares | F |
|---|---|---|---|---|
| Regression | 1 | 2222.9 | 2222.9 | 6.65* |
| Residual | 8 | 2674.8 | 334.3 | |

*P = 0.033